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The Role of Cyclooxygenase-2 (Cox-2) in the Proliferation and Differentiation of Osteoblastic Cells

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THE ROLE OF CYCLOOXYGENASE-2 (COX-2) IN THE PROLIFERATION AND DIFFERENTIATION OF OSTEOBLASTIC CELLS

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Table of Contents

List of Tables

List of Figures

Review of Literature

Introduction

Bone remodeling

The maintenance of bone mass in the skeleton depends on bone turnover. Bone turnover is the result of many cycles of bone resorption and formation occurring locally throughout the skeleton. Cycles are initiated by the arrival of bone resorbing cells, osteoclasts, which resorb a packet of bone and then leave the scene probably via apoptosis or programmed cell death. Osteoclasts are followed by bone forming cells, osteoblasts, which lay down matrix proteins, mostly type I collagen, to replace the bone that has been resorbed. This cycle is "coupled" because formation always follows resorption as long as there is a template remaining. Orthodontically applied forces attempt to manipulate this cycle and to remodel the bone surrounding a tooth such that the tooth will be in a more favorable location.

Orthodontic tooth movement is generally explained by the pressure-tension hypothesis, in which bone is resorbed on the areas under pressure and deposited at sites of tension. [1] When a force is applied to the tooth, the tooth moves immediately 0.2-0.3 mm due to the compression of the periodontal ligament (PDL). The tooth remains in that position, for several days, during which localized necrosis occurs in the compressed PDL. Osteoclastic resorption that occurs in the bone marrow space adjacent to the area of necrosis is called "undermining" bone resorption. After the removal of the necrotic PDL, bone resorption in the area of compression occurs in the periodontal space. Once this

frontal bone resorption is initiated, the tooth begins to move (Figure 1). [2] Biochemical mediators that are induced in the surrounding cells further complicate the process of tooth movement. For example, the cells of the compressed periodontal ligament secrete cytokines, which stimulate osteoclastic formation and resorption in the direction of the orthodontic force. [3] These cytokines not only stimulate bone resorption, but also stimulate the production of prostaglandins (PGs), which are themselves potent stimulators of resorption. [3] Applied mechanical load during tooth movement can also induce prostaglandin production. [4] Prostaglandins have been shown to enhance tooth movement under orthodontic forces, presumably by enhancing bone resorption. [4-8] However, prostaglandins can also regulate bone formation and the integrity of the newly remodeled bone surrounding the tooth will depend on the formation response to resorption. Thus some of the sources of prostaglandin production may be from compressed and fluid stressed cells in the PDL after orthodontic loading as well as from cytokine stimulation from necrotic PDL tissues.

Bone Formation

For each new cycle of bone turnover, osteoblasts differentiate from multipotential mesenchymal stem cells, in the bone marrow, into a series of precursor cells. [9] The mature functioning osteoblast makes type I collagen, the major component of bone matrix. It also produces bone specific non-collagenous proteins that are important for mineralization. Consistent with its anabolic function, the osteoblast is characterized by a great quantity of granular endoplasmic reticulum (ER), an abundance of free ribosomes, a well-developed Golgi apparatus, and numerous mitochondria. [10] Once the osteoblast

has completed its function, it can take one of three pathways. It can become an osteocyte, a spidery cell that is trapped in mineralized bone, which cannot replicate further. It can also become a lining cell, which covers the mineralized bone surface. Finally, the osteoblast can undergo programmed cell death or apoptosis. [11]

According to studies by Stein and Lian [12], there are three very distinct and sequential stages in the progression of an osteoblastic progenitor cell to a mature osteoblast. The first stage involves a period of rapid proliferation during which the extracellular matrix (ECM) is synthesized and the number of cells presents increases. When proliferation slows, precursor cells can then differentiate into cells that make proteins characteristic of mature osteoblasts, such as osteocalcin and type I collagen. At this point, the ECM undergoes development, organization, and maturation. Finally, the ECM undergoes mineralization (Figure 2). [12]

The study of differentiation of osteoblastic precursors into mature osteoblasts can be conducted using cultures of bone marrow cells or cultures of cells enzymatically digested from calvariae. These primary calvarial cultures are thought to include a small population of osteoblastic precursors. [13, 14] There are several generally accepted markers of osteoblast proliferation and differentiation. The initial stage is characterized by the production of cell cycle (histone) and cell growth (c-myc, c-fos, c-jun) related genes that encode proteins that enhance active proliferation. Also, other genes associated with the formation of the ECM such as type I collagen, are actively produced. [12] The second stage is characterized by the expression of alkaline phosphatase (ALP), an early marker for colony forming units-fibroblastic (CFU-f) units that can differentiate to become osteoblasts. [12] Osteocalcin expression is a marker for the third period,

matrix maturation, and corresponds with the increase in total mineral accumulation. [12] Another marker of osteoblastic mineralization is the deposition of hydroxyapatite crystals indicated by the von Kossa silver staining of the mineralized nodules. [15] The process of osteoblastic proliferation and differentiation is regulated by many factors including cytokines, mechanical stress, hormones, growth and differentiation factors, and cell-cell and cell-matrix interactions. [16]

The osteocyte, the most abundant cell in the human bone, is about ten times more prevalent than the osteoblast. [17] It is an "end stage" cell that can no longer proliferate like the osteoblast but can make and secrete factors that can influence bone turnover. Osteocytes are contained in a lacunar-canalicular system of bone in which the cell body is contained in the lacuna and the cytoplasmic processes are housed in the canaliculi. The osteoblast's progression to an osteocyte begins with a signal, possibly from another recently embedded osteocyte, which stimulates an osteoblast to commit to osteocyte differentiation while simultaneously directing a preosteoblast in the same area to differentiate into a mature osteoblast to replace the former. [18] The osteocyte is characterized by a decrease in the number of organelles, a reduction in the cytoplasmic volume, and cellular body volume. [10] In addition, it acquires long slender processes, which allow the osteocyte to communicate with other embedded osteocytes, as well as with the lining cells [19] via gap junctions found on the cell bodies and on the cytoplasmic processes. [20, 21]

As yet, the functions of osteocytes have not been conclusively determined. However, one theory that is gaining credence is the possible role of osteocytes as mechanosensory cells. Cowin et al. [22, 23] as well as Burger and Klein-Nulend [24]

proposed that osteocytes are mechanically activated by the flow of interstitial fluid through the lacuno-canalicular system (Figure 3).

Bone Formation in Alveolar Bone

The proliferation and differentiation of osteoblasts in the alveolar bone begin in the periodontal ligament (PDL). The PDL is a connective tissue interface between a tooth and its surrounding bone. It is largely composed of three substances: 1) collagen fibers, 2) ground substance which is a gel-like matrix mainly of glycoproteins, proteoglycans, and hyaluronan, and 3) fibroblast-like cells (FLC) (Figure 4). FLC constitutes fifty percent of the PDL by volume [25] and contain a population of both osteogenic and non-osteogenic cells. [26] The FLC in the PDL eventually give rise to osteoblasts, cementoblasts, fibroblasts, and macrophages, as well as others that have not yet been determined. [27] Roughly half of the FLC in the rat PDL contributes to the population of osteoblasts. [28]

The active cell cycle is divided into four phases: (1) G_1, a period of pre-DNA synthesis, (2) S phase, a time in which DNA replicates, (3) G_2, a period of post-DNA synthesis, and (4) M, the period of mitosis in which the cell divides producing two identical daughter cells. [27] During the period of active proliferation, the approximate duration of each phase is 21, 9, 2.5, and 1.2 hours respectively. [29] Non-cycling cells may arrest at two points in the cell cycle. The first point, at the end of G_1 and prior to S phase, is referred to as G_1 blocked. The second point, at the end of G_2 and prior to mitosis, is called G_2 blocked. Reserve cells in deep G_1 block are referred to as G_0 cells (Figure 5). [27]

Roberts and Chase studied osteogenesis in the PDL following mechanical stimulation. They inserted orthodontic elastic between the upper first and second molars of the rat causing compression both in front of the first molar and behind the second molar. They concluded that cells with osteogenic potential were originally dispersed within the PDL. However, with the onset of mechanical stimuli, these cells began to proliferate and differentiate into osteoblasts as they migrated to the bone surface. [30] Specifically, there is a cyclic decrease, increase, and return to basal levels of S phase cells within 15 minutes to 2 hours as measured by ^{3}[H]-thymidine labeling index. [31] Throughout the PDL, mitotic bursts were recorded as early as 2 hours and elevated labeling indices were shown as early as 6 hours following a mechanical stimulus. Given that the labeling and mitotic activity occurs within hours of the stimulus, it is believed that there is a transient release of quiescent G_1 blocked (G_0 reserve cells) and G_2 blocked cells in the PDL. [32] In addition, since only about half of the initial group of osteoblasts was labeled with ^{3}H thymidine despite its availability, it indicated that there were preosteoblasts already in the PDL that were destined to immediately progress into osteoblasts. [30]

A more localized response follows at about 12 hours to several days post-stimulus. This specific osteogenic response is prolonged and the cell proliferation is localized to the immediate area of new bone formation. Peak DNA synthesis occurs at about 20 to 22 hours and peak mitotic activity occurs at about 30 hours. Roberts et al reasoned that these osteoblasts, due to the time delay, resulted from passage through the cell cycle. [29]

Prostaglandins

Prostaglandins (PGs) are potent and complex regulators of bone metabolism. PGs, abundant in bone, are produced primarily by osteoblasts and are derived from phospholipids in the membrane bilayer. Phospholipase A_2 (PLA_2) releases arachidonic acid (AA) from the membrane phospholipids. [33] Prostaglandin G/H Synthase (PGHS) or cyclooxygenase (COX), the rate limiting enzyme in the conversion of AA to prostanoids [34], converts AA to PGG_2 via a cyclooxygenase reaction. The same enzyme then reduces PGG_2 to PGH_2 in a peroxidase reaction. Finally, tissue specific intracellular enzymes convert PGH_2 into the various prostaglandins (PGE_2, PGD_2, PGI_2, and PGF_{2a}) (Figure 6).

Two forms of COX have been discovered, COX-1 and COX-2. [35] Both are located in the endoplasmic reticulum and the nuclear envelop and both have all the structural features necessary for catalytic functions. [36] However, there are many differences between COX-1 and COX-2. Although these enzymes catalyze the same reaction, they are encoded by separate genes [37, 38] and are expressed and regulated differentially. In addition, COX-1 and COX-2 may be able to use endogenous and exogenous arachidonic acid differentially [39] and may be functionally coupled to different trans-membrane phospholipases. [40] Most importantly, it has been established that COX-1 gene expression is generally constitutive while COX-2 is inducible.

COX-1 is thought to produce prostaglandins necessary for daily "housekeeping" functions such as renal blood flow maintenance, gastric cytoprotection, and platelet aggregation. COX-1 gene expression is only moderately affected by the same factors that affect COX-2 expression. [33, 34, 41] In contrast, COX-2 is considered to be the

synthase involved with inflammation and other acute responses. As such, it is often undetectable in tissues at basal conditions but can be rapidly and transiently induced to high levels by multiple factors. These factors include cytokines IL-1 [42-46], TNF_{α} [44, 46], and IL-6 [47]; growth factors TGF_{β} [42, 48], TGF_{α} [42, 45], and FGF-2 [49]; hormones like parathyroid hormone (PTH) [43, 50, 51]; prostaglandins [43, 50-52] and fluid shear stress. [24] Serum is also a stimulator of prostaglandin in cultured osteoblasts. [48] Glucocorticoids [43, 48], retinoic acid [53], and the cytokines IL-4 and IL-13, [44, 54] inhibit COX-2 expression. COX-2 is highly expressed in bone and appears to be the enzyme responsible for most prostaglandin responses in bone including inflammation. [35]

In addition, COX-2 is thought to be involved with mitogenesis and cellular differentiation. [55] The scientific literature has clearly documented that the continuous use of NSAIDs has reduced the risk of colorectal cancer approximately 50% in humans. [56-58] Not surprisingly, there was an increase in COX-2 expression in human colorectal adenocarcinomas [59] and elevated levels of COX-2 proteins in colorectal tumors. [60] One study used APC $^{\Delta 716}$ mice, which develop hundreds of tumors in the intestines, and bred these mice to COX-2 KO mice. The result was an 80-90% reduction of tumors in the COX-2 KO mice. [61] In addition, when the APC $^{\Delta 716}$ mice were given a highly selective COX-2 inhibitor (SC-58125), there was a significant reduction in tumors. [61] Dubois et al. concluded that over-expression of COX-2, often seen in colorectal cancer, prolonged the G_1 part of the cell cycle. By greatly increasing the time in G_1 and inhibiting cell apoptosis, COX-2 over-expression could increase the length of time that abnormal cells would exist and increase the chance for further mutations of that cell.

Thus the prolonged survival of abnormal cells can favor sequential gene mutations that could result in tumor development. [62] Others have shown that COX-2 inhibition may induce apoptosis [63] while the addition of exogenous PGE_2 reversed the induction of apoptosis produced by COX-2 inhibitors. [64]

Prostaglandins and Bone Formation

The regulation of bone formation by prostaglandins appears to be quite complex. The scientific literature is controversial in regards to the effects of prostaglandins on the proliferation of osteoblasts. Some studies suggest that prostaglandins enhance proliferation. In one study, PGE_2 was added to cultures of primary osteoblast cells from embryonic chick calvariae in the concentrations of 10^{-8} to 10^{-5} M. PGE_2 caused a dose related increase in proliferation as measured by ^{3}[H] thymidine incorporation and total DNA content of the cultures. [65] Another study by Chyun et al. showed that PGE_2 at 10^{-7} M stimulated both DNA and collagen synthesis as shown by ^{3}[H] thymidine and ^{3}[H] proline incorporation. In addition to its stimulatory effects, PGE_2 also reversed the inhibitory effects of cortisol to levels above control untreated cultures. [66]

Other investigators have found conflicting results. In UMR 106-01 rat osteosarcoma cells, Fang et al. found that PGE_2 inhibited mitogenesis. [67] Another group reported that PGE_2 at 10^{-6} M decreased proliferation of the human osteoblastic osteosarcoma cell line G292 and Saos-2. [68] Fujimori et al. reported that indomethacin stimulated cell growth in MC3T3-E1 cells by blocking endogenous PGE_2 synthesis. [69] Interestingly, the same group later demonstrated a bi-phasic role of prostaglandins in osteoblast proliferation. They found that while exogenous prostaglandins inhibited cell

proliferation at 6×10^{-8} M and 6×10^{-7} M, in the osteoblastic cell line MC3T3-E1, there was also a slight stimulation of cell growth at 6×10^{-6} M. The addition of cyclooxygenase inhibitors, such as acetyl salicylic acid (ASA), fluriprofen, and piroxicam, enhanced cell growth. These results suggest that the cyclooxygenase inhibitors may stimulate growth by inhibiting the production of endogenous prostaglandins. [70] Baylink et al. also found biphasic effects of prostaglandin on osteoblast proliferation. However, in contrast to Fujimori's studies, they found stimulation of osteoblastic proliferation at lower concentrations (10^{-9} M) and inhibition at higher concentrations (10^{-6} M). [71]

The data on the effects of prostaglandin on the differentiation of osteoblasts are more conclusive. Prostaglandins clearly enhance differentiation of osteoblastic progenitors into mature osteoblasts. [10-12] Scutt et al. showed that the addition of PGE_2 to the cell cultures of marrow stromal cells increased osteoblast differentiation without a corresponding increase in proliferation. [72] In rat calvarial osteoblasts, Kanecki et al. found that PGE_2 inhibited proliferation but stimulated differentiation. [73] Still another investigator found that PGE_2 stimulated both the proliferation and differentiation of osteoblastic MC3T3-E1 cells. [74, 75] Despite the conflicting reports regarding the effect of PGE_2 on proliferation, the majority of the literature agrees on the effect of PGE_2 on differentiation. Interestingly, although prostaglandins stimulate the differentiation of osteoblasts, they generally inhibit the function of mature osteoblasts, such as the ability to make collagen. [76, 77]

Knockout Mice

Prostaglandins are a diverse group of autocrine and paracrine hormones that are involved with many cellular and physiologic processes. In order to study the effects of PGs, NSAIDs were used to inhibit its production. However, since different NSAIDs affect both COX-1 and COX-2 to varying degrees [78], it was difficult to establish whether or not the results of the study were due to the inhibition of COX-1 or COX-2. Transgenic mice, in which the COX-1 or COX-2 are disrupted, have been produced by insertion of a neomycin cassette in the location of the COX-1 or COX-2 gene. [79-81]

COX-1 deficient or knock out (KO) mice develop normally and appear to be healthy. Examination of the liver, spleen, kidney, gastrointestinal tract, reproductive organs, heart and lungs reveal no major pathology. In some of the kidneys of the COX-1 KO mice, there were several areas characterized by immature tubules but the size and frequency did not increase with age. They have no gastric pathology, show decreased indomethacin-induced gastric ulceration compared to wild-type (WT) mice, and have a reduced inflammatory response to exogenous arachidonic acid (AA). In addition, COX-1 KO mice have a reduced capacity for platelet aggregation in response to AA. Due to parturition difficulties, breeding between COX-1 KO mice produce few live offspring. [79]

COX-2 KO mice, on the other hand, may have a shorter lifespan primarily due to renal abnormalities. Examination of the organs of the COX-2 mice revealed kidneys, which were small, pale, and granular in appearance. Some areas showed immature glomeruli while others showed areas of inflammation and atrophy. The severity of renal neuropathy increases with age. The kidneys of 3-day-old COX-2 mice did not differ

from the kidneys of the WT mice. Thus it suggests that due to the presence of some PGE_2 in utero from the mother or other embryos, the development of the kidneys in COX-2 mice occurred normally. However, with the birth of the COX-2 mice, the lack of PGE_2 prevented the further development of the kidneys resulting in renal problems, which increased over time. [80] In addition to renal pathology, sporadic cardiac fibrosis and increased susceptibility to peritonitis characterize COX-2 KO mice. COX-2 KO females have multiple problems in the reproductive process, including absent ovulation, due to the absence of the corpora lutea. Difficulties in fertilization, implantation, and decidualization have also been noted. [82] Despite these problems, COX-2 mice have a normal inflammatory response to AA and tetradecanoyl phorbol acetate (TPA) as well as a normal inflammatory response to bacterial invasion of the peritoneum. [80]

COX-2 heterozygous (HT) mice survive and breed normally. Due to the difficulties of producing live offspring from the breeding of female COX-2 KO mice, [79, 80] knockout mice are obtained from crossing of HT mice.

Prostaglandins and Tooth Movement

In general, orthodontics tooth movement is considered to be a localized and controlled area of inflammation. As discussed above, the reaction to the forces placed on the tooth results in the release of such factors as prostaglandins, which results in the inflammation process. As PGs are postulated to stimulate resorption and are generally considered to facilitate inflammation, the rate of tooth movement should increase with PG administration. In 1990, Lee showed that the administration of PGE_1 to rats, both locally and systemically, enhanced bone resorption. [5] Davidovich et al. went one step

further to show that the levels of PGE_2 increased in alveolar bone of cats during tooth movement. [4] Yamasaki et al. showed a two-fold increase in the rate of tooth movement with the injection of PGE_1 and PGE_2 in monkeys. [7] In limited clinical trials, buccal injections of PGE_1 produced 1.6-2 times more tooth movement when compared with controls with minimal side effects. [8] Further studies showed that lower concentrations of PGE_2 (0.1 or 1.0 micrograms) were as effective as higher concentrations (5.0 and 10.0 micrograms) in enhancing tooth movement and that rats with single injections of PGE_2 showed similar results as rats with multiple injections. [83, 84]

Numerous studies showed that PGs enhance the rate of tooth movement. It would then seem logical that inhibiting the production of PGs would slow the rate of tooth movement. Nonsteroidal anti-inflammatory drugs (NSAIDs) are a class of agents that have anti-pyretic, anti-inflammatory, and analgesic properties. They act by blocking the production of PGs, and thereby reducing inflammation (Figure 7). [85] The older NSAIDs inhibit both COX-1 and COX-2 differently. For example, aspirin irreversibly inactivates both COX-1 and COX-2 but its effects are more profound on COX-1. [86, 87] Aspirin also inactivates COX-2 by catalyzing the production of 15-HETE instead of PGH2. [88, 89] Ibuprofen acts by reversible inhibition of both isoforms about equally while the active metabolite of nambutone preferentially inhibits COX-2. [55, 90] In general, the older non-selective NSAIDs have anti-inflammatory properties but tend to have gastrointestinal side effects. [91] COX-2 selective inhibitors have anti-inflammatory properties with little gastrointestinal effects. [92, 93] There are two new COX-2 inhibitors on the market, Vioxx ™ and Celebrex ™. Since NSAIDs are routinely used for

the treatment of orthodontic discomfort, its effect on tooth movement would be relevant to the clinician.

Indomethacin, a potent inhibitor of PG synthesis, is often used in the study of prostaglandin inhibition. In one study, indomethacin was administered perorally to miniature pigs undergoing tooth movement for a 39-day observation period. Histology of the bone revealed that the extent of resorption was significantly reduced. Formation surfaces of bone were also reduced but not significantly suggesting that the rate of bone turnover but not remineralization may be influenced by indomethacin. [94] In another study, cats were given indomethacin orally and observed for 21 days. The rates of tooth movement for the treated cats were two times slower that for the controls. Therefore, NSAIDs may be contraindicated for patients undergoing orthodontic treatment. [95]

Hypothesis

We hypothesize that endogenous PGs produced by the induction of COX-2 act to switch osteoblastic cells from the proliferative state into differentiating state. Therefore, we expect osteoblastic cell cultures from COX-2 knockout mice (KO), in which the COX-2 gene has been disrupted, to proliferate more rapidly than cells from wild type mice with normal COX-2 gene expression (WT). We also expect osteoblastic cells from COX-2 knockout (KO) mice to differentiate less rapidly than cells from wild type (WT) mice. In addition, if exogenous PGs are added to the COX-2 cell cultures, the differences between wild type (WT) and knockout (KO) cells should be reversed.

Objective

The purpose of this research is to determine the role of prostaglandins (PGs) in the proliferation and differentiation of primary calvarial osteoblasts from COX-2 knockout and wild type mice.

Specific Aims

1) To test the hypothesis that osteoblastic precursor cells from COX-2 knockout (KO) mice proliferate more rapidly than those from COX-2 wild type (WT) mice and that the

difference is due to the lack of PGE_2 production. We will use primary osteoblastic cells obtained by enzymatically digesting calvariae from COX-2 KO and WT mice. We will measure cell count using a Coulter Counter on sequential days of culture, beginning on Day 3 and continuing until cell numbers have reached a plateau. We will measure PGE_2 in the culture medium by Radioimmunoassay (RIA) to document differences in PGE_2 production by WT and KO cells. We will treat KO cells with exogenous PGE_2 (0.1 to 1.0 μM) to see if we can reverse the differences.

2) To test the hypothesis that osteoblastic precursors from COX-2 knockout mice differentiate more slowly than those from COX-2 wild type mice and that the difference is due to the lack of PGE_2 production. We will use primary osteoblastic cells obtained by enzymatically digestion of the calvariae from COX-2 KO and WT mice. We will measure markers of osteoblastic differentiation by the following methods: A) staining for alkaline phosphatase (ALP) on sequential days of culture beginning on Day 6 B) Measure ALP activity at the end of the first and second weeks of culture. We will measure PGE_2 in the culture medium by RIA to document differences in PGE_2 production by WT and KO cells. We will treat COX-2 KO cells with exogenous PGE_2 (0.1 to 1.0 μM) to see if we can reverse the differences.

Materials and Methods:

Breeding and Genotyping

As homozygous COX-2 KO female mice are infertile, heterozygous (HT) COX-2 mice were bred to obtain the COX-2 KO mice. The litters were typed using polymerase chain reaction (PCR) amplification of DNA taken from tail snips. Litters contained three genetic types: wild type (WT), heterozygous (HT), and knockout (KO). Each calvariae were dissected and the primary osteoblast cells were harvested in the manner stated below.

Primary Osteoblast (POB) Cell Culture

Whole calvariae were dissected from 5-12 week old COX-2 (WT, HT, KO) littermates, and dissected free of loose connective tissue. After rinsing with PBS, the calvariae were sequentially digested with 0.5 mg/ml crude collagenase and 0.01% trypsin- 0.5% mM ethylenediaminetetraacetic acids (EDTA) for 10 minutes at 37^0 C, with the exception of fifth digest which will be for 45 minutes. The enzymatic reaction was stopped by 10% heat-inactivated fetal calf serum and the released cells were filtered through a Nitex membrane to obtain a single cell suspension. As the first digest contained a mixture of cells including fibroblasts [96, 97], it was discarded and only digests 2-5 were saved. The cells from digests 2-5 were pooled, spun, resuspended, and plated in 100 mm culture dishes in Dulbecco's modified Eagle's medium without phenol red, 10% heat-inactivated fetal calf serum (HI-FCS), penicillin (100 U/ml), and streptomycin (50 μg/ml).

These cells were grown to confluence, with the culture medium to be changed every 3-4 days. Once the cells were confluent, the cells were trypsinized from the culture dish, split, and plated into 6 well dishes at 5000 cells/cm^2. The same medium, with the addition of 50μg/ml phosphoascorbic acid (L-ascorbic acid phosphate magnesium salt; Wako Pure Chemical Industries, Japan), was used for plating of these cells. The cells were cultured up to 4 weeks with medium changes every 3-4 days.

For the prostaglandin studies, different concentrations of prostaglandin (PGE_2) were pulsed into each well at the time of plating. The control wells were similarly pulsed with ethanol. Subsequent medium changes already had the prostaglandin incorporated into the medium, thus eliminating the need for the addition of prostaglandin into each well. In addition to the medium change every 3-4 day, 1 ml of the old medium was collected from the control wells and frozen until use for RIA.

Cell Count

Each well was rinsed with PBS and trypsinized with trypsin-EDTA. Cells from each well were collected into a 15-ml centrifuge tube. The cells were centrifuged and were resuspended in 500 μl of DMEM solution. An aliquot of 100 μl was taken from the dispersed cell suspension and placed in a counting vial with 9.9 ml of the Coulter Counter buffer solution. If there was a large pellet after centrifugation, the cells were resuspended in 1000 μl of DMEM solution with a subsequent 100-μl aliquot for counting. The lower limit set for the Coulter Counter was 12.0 μm. Before counting and in between the different groups, several counts were made on the buffer solution as a control. The Coulter Counter displayed a reading as the number of cells per ml. If the resuspension

occurred in 500 µl of solution, then the true number of cells per well was half of what was displayed. If the resuspension was in 1000 µl of solution, then the displayed number was the true number of cells per well.

Alkaline Phosphatase (ALP) Staining

Alkaline phosphatase staining was done with a commercial staining kit (Sigma). The cells in each well were rinsed with PBS, fixed with a solution of citrate solution, acetone, and formaldehyde for 30-60 seconds. The cells were rinsed in water and the excess moisture was removed with a pipette. The alkaline phosphatase stain, a solution of 8.3% FRV, 8.3% napthol, 8.3% nitrite solution, and 75% water, was prepared from a commercial kit. (Sigma) The stain remained on the cells for 15 minutes, in the dark. After incubation, the cells were rinsed with water and allowed to air dry.

Alkaline Phosphatase (ALP) Activity

After the culture medium was removed, cells were rinsed three times with PBS and scraped in 0.5 ml of CAT scraping buffer (40 nM Tris pH 7.4, 1 mM EDTA, 0.15 M NaCl). The cells were centrifuged for 30 seconds and the supernatant was removed. The cells were resuspended in 100 ml of lysis buffer (10 mM Tris-HCL buffer, pH 7.5, plus 0.1% Triton X-100). The samples were subjected to 3 freeze-thaw cycles on dry ice and centrifuge for 5 minutes. 10 µl of the supernatant were transferred into micro titer wells and the sample volume was brought up to 20 µl with lysis buffer. 180 µl of the assay mix containing 2 mM p-nitrophenol phosphate as substrate and 2 mM $MgCl_2$ were added to the sample wells and incubated at 37 ^{0}C until the color developed (2 minutes to 2 hours).

The absorbance was read every 15-min at 410 nM for an hour. AP activity was determined relative to a standard curve, normalized to the soluble protein content of the extracts and expressed as nmol/min/mg protein. Histochemical assessment of AP activity was carried out on unfixed cultures using FRV-alkaline solution and fast red violet LB base in HCl (Sigma protocol No. 86). After incubation for 30 min at room temperature, cells were rinsed with PBS and fixed in methanol.

Prostaglandin Radioimmunoassay (RIA)

Medium was removed from the cultured cells prior to changing the medium and frozen until use. PGE_2 accumulation was measured using Radioimmunoassay (RIA). [98] RIA for PGE_2 was carried out using antibodies provided by Dr. Laurence Levine of Brandeis University. Unknown and standards contained equal amounts of BGJ-BSA. ^{3}[H]-prostaglandins were used as tracer and the assays were run at antibody dilutions providing 20-40% binding. The lower detection limit of the assay for PGE_2 was approximately 5 pg per tube or 0.14 nM in a 100 µl sample, and the intra assay variation was 6%. The assays were carried out at 4 ^{0}C and free prostaglandins were moved with dextran-coated charcoal. The values for unknowns were calculated from a standard curve using the logit-log, curve-fitting computer program (simple RIA program, D. Rodbard, BCTIC, Computer Code Collection, Nashville, TN). The data were presented as mean, standard error, and significances of differences determined by student's t-test. The data were presented as pg of PGE_2/ml of medium.

DNA Content and ^{3}[H]-thymidine incorporation

Cells grown in six-well dishes were washed with PBS, extracted with 10 % trichloroacetic acid, and digested overnight with 0.5 M NaOH at 4 ^{0}C. Aliquots were neutralized with 0.5 M acetic acid, and after the addition of diaminophenylindole, DNA content was determined fluorometrically (Fluorolite 1000, DynaTech Laboratories, Chantilly, VA). The DNA content was calculated as micrograms of DNA per well for six wells, and values were expressed as the mean ± SEM. [42] ^{3}[H]-thymidine incorporation was calculated as dpm/micrograms of DNA per well for six wells, and the values were expressed as the mean ± SEM.

Statistical Analysis

The statistical significance of the differences among the means was determined by analysis of the variance (ANOVA), with post-hoc comparison of more than two means by the Bonferroni method. The computer program Sigma Stat was used to run the statistics.

Results

Phenotype of WT and KO Mice

The COX-2 WT (+/+) and KO (-/-) mice were 5-12 weeks old and appeared to be healthy and active. The skeleton appeared to be normal and there was no significant difference in size or weight (Table 1). No difference was found in the bone histology for 5-week-old COX-2 WT and KO mice (data not shown). In addition, there was no statistical difference in serum creatinine levels indicating that kidney function in both the WT and KO mice was normal (Table 1).

Cell Count: Comparison of COX-2 WT and KO primary osteoblastic cell cultures

To evaluate the differences in the cell count between cultures with and without COX-2, primary osteoblastic cells enzymatically digested from calvariae (pooled populations 2-5) from COX-2 WT and KO mice were plated at a density of 5000 cells/cm^2. The cells were cultured for a total of 6 days; with the cell counts measured on days 2, 3, 5, and 6 (Figure 8). At day 2, the WT cultures were significantly increased by cell count relative to the KO cultures ($p < 0.01$). At day 3 however, the cell count from the KO mice was 1.3 times higher than the cell count from the WT mice ($p < 0.01$). The increased cell count of the KO cultures compared to the WT was also found on days 5 and 6 ($p < 0.01$). Other experiments confirmed this increase in cell count in the KO cell cultures relative to the WT cell cultures (Table 2). Seven cell count comparisons between WT and KO cultures were made in four different experiments between days 5

and 8 of cell culture. Six out of the seven comparisons showed a 1.2 – 4.5 fold higher cell count in the KO cultures than the WT cultures ($p < 0.05$).

Despite the consistently higher cell counts in the KO group, there was a wide variation in the cell counts between experiments for the same day of culture even though initial plating density was the same for all experiments. One explanation for this differential rate of cell growth among the experiments may have been due to the different ages of the mice (5-12 weeks) from which the calvariae were harvested. It was possible that the primary osteoblastic cells digested from the calvariae of younger mice grew more quickly. However, the differential cell counts among the experiments appeared to have no direct relationship to the ages of the mice (Table 2). In fact, cultures from younger mice appeared to grow more slowly.

To evaluate the differences in the cell count between cultures with varying amounts of COX-2, we measured the cell counts from COX-2 WT (+/+), HT (+/-), and KO (-/-) cell cultures. The cells were cultured for 3, 4, 6, and 7 days (Figure 9). Throughout the 7 days of culture, the KO cultures had significantly greater cell counts than the WT cultures ($p < 0.01$). The HT cultures had significantly lower cell counts than the KO cultures at day 6 ($p < 0.05$), and higher cell counts than the WT cultures on days 3 ($p < 0.05$), day 6 ($p < 0.01$), and day 7 ($p < 0.05$). Thus, the HT response appeared to be intermediate between that of the WT and KO cultures.

Role of PGE_2 in the cell count difference between WT and KO primary osteoblastic cell cultures

We have previously shown [48] that fresh serum-induced expression of COX-2 and COX-2 associated PGE_2 production in cultures osteoblasts. To determine if the absence of COX-2 expression resulted in the difference of endogenous PGE_2 in WT and KO cultures, the medium of the cell cultures were sampled on days 3 and 7 and radioimmunoassay (RIA) was conducted to measure the levels of PGE_2 (Figure 10). On day 3, PGE_2 in the medium of the WT cell culture was 70 ± 2.2 nM/well but levels in the medium of the KO cell culture undetectable (< 0.1 nM). Culture medium with fresh serum was replaced after measurement on day 3. On day 7, the levels of medium PGE_2 in the WT cell culture were reduced by about 70 % as compared to day 3. Once again, the levels of medium PGE_2 in the KO cell cultures were undetectable. For both day 3 and day 7, the differences between the levels of medium PGE_2 between the WT and KO cell cultures were significant ($p < 0.01$). The decreased PGE_2 levels on day 7 probably reflected the decreased ability of fresh serum to stimulate COX-2 expression with increasing days of culture, a relationship that we have observed previously (data not shown).

To study the effects of exogenous PGE_2 on the cell count, primary osteoblastic cells from COX-2 WT and KO mice were cultured for 7 days. Beginning from the day the cells were plated, both WT and KO cells were treated with vehicle or PGE_2 (10^{-6} M). Fresh PGE_2 and vehicle were added when the media was changed. Cell counts were measured on days 3, 4, 6, and 7 (Figure 11). Throughout the 7 days of culture, there were no differences in cell count between the WT control and the WT cultures treated with PGE2 (10^{-6} M) cultures. PGE_2 completely reversed the increased cell count seen in the KO cultures on day 3. The cell counts from KO cultures treated with PGE_2 were 40%

less than the cell counts of the KO group on days 4 and 6 ($p < 0.01$) as well as for day 7 ($p < 0.05$). The cell counts from KO cultures treated with PGE_2 were significantly higher than the WT control group on all days except day 7 ($p < 0.01$). Thus, the addition of PGE_2 (10^{-6} M) to the KO cultures significantly reduced the cell number ($p < 0.01$) but had no effects on WT cultures. However, the exogenous PGE_2 at 10^{-6} M only partially reversed the increased cell count in KO cultures.

Comparisons of the above data with two other experiments showed the mixed results (Table 2). As stated above, experiment 14261 showed that PGE_2 partially reversed the increased cell counts of the KO cultures for day 6 and 7 ($p < 0.05$). Conversely, results from experiment 14369 showed that, for day 8, PGE_2 did reduce cell counts of the KO cultures but it was not significant. Finally, results from experiment 14533 showed no effect of PGE_2 on KO culture's cell count for day 5 and 6. In fact, there was even a slight increase in the cell count of the PGE_2 treated KO cultures on day 6. All three experiments did show a significant increase in cell count for KO cultures relative to WT cultures ($p < 0.05$) with the exception of experiment 14533 at day 6.

As noted in the literature, exogenous PGE_2 has a complex effect on osteoblastic proliferation, with bi-phasic effects dependent on the concentration of PGE_2 [70]. Hence, we examined the effects of a lower concentration, 10^{-7} M, of PGE_2. Primary osteoblastic cells from COX-2 WT and KO mice were cultured for 6 days. Beginning from the day the cells were plated, both WT and KO cells were treated with PGE_2. Fresh PGE_2 and vehicle were added when the media was changed. Cell counts were measured on days 2, 3, 5, and 6 (Figure 12). PGE_2 (10^{-7} M) had no effect on cell count in the cell counts from the WT cultures except for day 3, which showed that the treated cultures had cell counts,

significantly lower than the untreated WT cultures. However, the increased cell count in the KO cultures relative to the WT cultures was completely reversed by the addition of PGE_2 at days 5 and 6.

Measurement of ^{3}[H]-thymidine incorporation in primary osteoblastic cell cultures from WT and KO mice

The differences in the cell numbers between the WT and KO cultures can be explained in two ways. The difference could be due to greater cell replication in the KO cell cultures or to more apoptosis in the WT cells cultures. Total DNA content was measured so that the ^{3}[H]-thymidine incorporation could be normalized to account for more or less DNA in some cultures. The measurement of ^{3}[H]-thymidine incorporation into the DNA estimates the number of cells passing through the S phase of the cell cycle and therefore the rate of cell replication (Figure 5). This is a method of evaluating the relative number of cells in the WT and KO cultures.

Total DNA content and ^{3}[H]- thymidine incorporation were measured for COX-2 WT and KO primary osteoblastic cell cultures on day 7 (Figure 13). Two different experiments showed that the KO cell culture had 2 and 3 times more DNA than the WT cell cultures ($p < 0.05$ and $p < 0.01$ respectively). This suggested that the KO cultures had more cells than the WT cultures, consistent with the cell counts. The measurement of ^{3}[H]-thymidine incorporation, however, showed no difference between the WT and KO groups. These results indicated that, at those particular time points, there were no differences in the rates of replication between the WT and KO cell cultures. In a third experiment, total DNA content and ^{3}[H]-thymidine incorporation were measured on day

5 (Figure 14). There was approximately double the amount of DNA in the KO control cultures relative to the WT cultures ($p < 0.01$). This was again consistent with the 1.8 time greater number of counted cells in the KO cultures as compared to the WT cultures. However, there was no difference in ^{3}[H]-thymidine incorporation between the WT and KO cell cultures. Thus there was no difference in rate of replication of the WT and KO cell cultures at this particular point in time.

The effects of exogenous PGE_2 on total DNA content and ^{3}[H]-thymidine incorporation were also measured on day 5 (Figure 14). On the day of plating, both WT and KO cell cultures received PGE_2 (10^{-6} M) and vehicle. Subsequent additions of PGE_2 and vehicle were given during media changes. There were no significant differences in the measurement of ^{3}[H]-thymidine incorporation between PGE_2 treated and untreated WT and KO groups. The treatment of the KO cultures with PGE_2 resulted in about 40 % reduction of total DNA relative to the control cell cultures ($p < 0.01$). However, there was no difference in cell count between the KO cultures treated with PGE_2 and untreated cultures on that day. As discussed earlier, Dubois et al. found that over expression of COX-2 increased the length of time a cell spends in G_1 stage of the cell cycle and inhibited apoptosis [62]. Another study found that inhibition of COX-2 might induce apoptosis [63]. However, these studies do not provide an explanation for the discrepancy between the data on DNA content and cell count. It is possible that other alterations in the cell cycle had occurred as a result of presence or lack of COX-2. One possibility is that the untreated KO cells were delayed in S phase of the cell cycle, in which DNA was duplicated. This would explain the similar cell counts of the PGE_2 treated and untreated KO cultures as well as the 2-fold level of DNA content in the KO control cultures.

Another possibility could be that PGE_2 treated KO cells were delayed in G_1, the stage prior to the synthesis of DNA. This could again provide an explanation for the similarity of cell count and the differences in DNA content.

Comparison of osteoblastic differentiation in WT and KO calvarial osteoblastic cell cultures

Alkaline phosphatase is a marker for early osteoblastic differentiation. In order to compare the differences in osteoblastic differentiation, alkaline phosphatase (ALP) staining was done on WT and KO cell cultures (Figure 15). On day 6, there was a clear difference in staining between the two groups. The WT cell cultures showed distinct evidence of alkaline phosphatase staining whereas the KO cultures did not show any evidence of staining. By day 9, the difference between the two groups was no longer so clear. It appeared that ALP staining, which measured differentiation, was delayed rather than prevented in the KO cultures. By as early as day 9, the difference in staining was no longer evident. In addition to the intensity of staining, there was a difference in staining pattern of the cultures in all experiments. The KO group showed more staining in the middle of the wells whereas the WT groups showed more uniform staining. One reason for this pattern of staining may be a result of the differences in proliferation between the WT and KO cultures. The rapid increase in cell count in the KO group may have led to a greater confluence in the center of the wells. As a result, the confluent cells in the center of the wells began to differentiate while the cells along the edges of the wells continued to proliferate thus leading to the ALP staining pattern described above.

ALP activity is a more quantitative measure of osteoblastic differentiation than ALP staining. ALP activity was measured for WT and KO cells cultured for day 7 and 14 (Figure 16, 18). Two different experiments showed that the levels of ALP activity of the WT cultures were 10-17 fold higher than the KO cultures on day 7. The difference in ALP staining at day 7 was consistent with the increase in ALP staining of the WT cultures in the first week of culture (Figure 15). At day 14, there was no difference in the levels of ALP activity for the WT and KO cultures in both experiments (Figure 16, 18). Again, this was consistent with the results of the ALP staining, which showed that by as early as day 9, the staining of both WT and KO cultures was roughly equivalent.

Effect of PGE_2 in the differentiation of WT and KO primary osteoblastic cell cultures

Because COX-2 KO cultures make little PGs as stated above, we added PGE_2 back to the cultures to see if we could reverse the effects of COX-2 KO on differentiation. ALP staining was done on cultures treated with PGE_2 (10^{-6} M) and vehicle on day 7 (Figure 17). As shown above, there was a marked difference in staining between the WT and KO control cell cultures. The addition of PGE_2 (10^{-6} M) to the KO cultures largely reversed this difference, producing similar levels of staining in both groups. As discussed above, staining appeared to be concentrated in the middle of the wells of the KO cultures.

In another experiment we examined ALP staining on days 7 and 14. There appeared to be a very slight increase in staining of WT cultures treated with PGE_2 for both days 7 and 14 (Figure 18). At day 7, there was a smaller difference in the intensity of ALP staining between the WT and KO control cell cultures than seen in the previous

experiments. The addition of PGE_2 (10^{-6} M) again increased the level of the staining in the KO cell cultures, which was even slightly more intense than the WT control and WT PGE_2 groups. By day 14, the intensity of the WT control, WT PGE_2, and KO control groups were about equivalent. However, the KO PGE_2 group continued to show a relatively greater degree of staining.

Interestingly, the addition of PGE_2 (10^{-7} M) showed a different pattern of ALP staining (Figure 19). At day 6, PGE_2 (10^{-7} M) only partially reversed the decreased staining in KO cultures relative to the WT cultures. By day 9, both treated and untreated KO cell cultures showed approximately the same levels of staining with each other. However, the staining in the WT PGE_2 groups was slightly more increased relative to the WT control cultures. Once again, the difference in pattern of staining between the WT and KO was present.

The effects of exogenous PGE_2 (10^{-6} M) on ALP activity were measured on days 7 and 14 in two different experiments (Figure 16). The addition of PGE_2 to the WT cultures increased the levels of ALP activity 2-3 fold on both day 7 ($p < 0.01$) and day 14 ($p < 0.05$) whereas the ALP staining which showed no differences in the quantity of staining between the WT control and PGE_2 groups on day 7 (Figures 17,18). The addition of PGE_2 to the KO cultures increased the levels of ALP activity 18 times for day 7 and 4 times for day 14 ($p < 0.01$). These results are consistent with the ALP staining (Figures 17,18), which showed that exogenous PGE_2 promoted staining and thus the differentiation of the KO cultures.

In another study, ALP activity was measured for days 7 and 14, and produced different results from the ones above (Figure 20). At day 7, the levels of ALP activity

for the WT cultures were 10 times greater than the KO cultures ($p < 0.01$). However, there was no difference in ALP activity between the WT and KO cultures at day 14. PGE_2 increased the ALP activity about 30% in WT cells on day 7 but reduced the levels about 30% on day 14. These differences between the treated and untreated groups were not significant and were consistent with the results of ALP staining which showed no difference in the intensity of ALP staining (Figures 17,18). On day 7, the addition of PGE_2 to the KO cells increased ALP activity approximately 5 times as compared to untreated KO cells ($p < 0.01$). On day 14, PGE_2 had no effect on ALP activity in the KO cultures, contrary to the staining which showed a slight increase in staining in the KO cultures with the PGE_2 (Figure 18). The similarities in the levels of ALP activity on day 14 may indicate that the differences in the ALP staining of the KO cells seen in the earlier days may disappear over a longer period of culture.

Discussion

In the past, the ways in which investigators studied the impact of prostaglandins on the proliferation and differentiation of osteoblastic cells were limited. In order to study the effects of the absence of PGs, NSAIDs were used to block the production of PGs. However, NSAIDs can have other effects on cells in addition to their inhibition of PG synthesis, which may have masked the effects of PG inhibition in these experiments. Recently, the introduction of mice with the genes for COX-1 or COX-2 disrupted has made it possible to study the role of PGs without the complications of the effects of NSAIDs. We focused on COX-2 KO mice because our lab has previously shown that COX-2, not COX-1, is the enzyme responsible for PG responses in bone cells.

Using primary calvarial cells from COX-2 WT and KO mice, we found that endogenous prostaglandins had profound effects on the proliferation of osteoblastic cells. We found, on average, a 2-fold higher cell count in the KO cultures compared to the WT. RIA of the medium from these cultures showed high levels of PGE_2 in the WT but undetectable levels in the KO cell culture medium. We concluded that the absence of COX-2, and therefore endogenous PGs, was associated with an increase in the cell count. The addition of PGE_2 had variable effects on the cell count, depending on the concentration. PGE_2 (10^{-7} M) completely reversed the increased cell counts in the KO to the level of the WT whereas PGE_2 (10^{-6} M) partially, but significantly, reversed the cell counts in the KO cultures. Since exogenous PGE_2 could reverse the effects of the absence of COX-2, it suggests that the higher cell counts in the KO cultures were a result of the absence of PGE_2 generated by COX-2.

Our studies have shown that there was an increase in cell counts in the COX-2 KO cultures. One explanation for the difference in cell count is an increase in the rate of replication by the COX-2 KO cells. As discussed earlier, the relationship between PGs and osteoblastic proliferation is complex. Previous studies in osteoblasts have shown that PGE_2 increased proliferation [65, 66]; decreased proliferation [67, 68], or both [70, 71]. Our studies showed no increase in the rate of replication for the COX-2 KO cultures on days 5 and 7. It is possible that we had missed the period in which the increase in replication occurred. In order to minimize the likelihood of missing this stage, we can measure the TDR at more frequent and earlier days of culture.

Another explanation for the increase in cell count in the COX-2 KO cells is that there was a decrease in apoptosis in the KO cells. However, data from non-bone cells suggest that COX-2 produced PGs, which decreased apoptosis. As discussed earlier, several studies have shown that the over-expression of COX-2 and, thus an over-abundance of PG's, led to a decrease in apoptosis. Dubois et al. stated that over-expression of COX-2, often seen in colorectal cancer, prolonged the G_1 phase of the cell cycle of rat intestinal epithelial cells by 3-fold. They concluded that by greatly increasing the time in G_1, the lifespan of the cell was extended and apoptosis reduced. [62] Sheng et al. stated that SC-58125, a selective COX-2 inhibitor, reduced colony formation of human colon cancer cells and that the inhibition of growth was reversed by the addition of PGE_2. Furthermore, they reported that PGE_2 inhibited the apoptosis caused by SC-58125. [64] Another study by Hanif et al. also found that NSAIDs, specifically sulindac sulfide and piroxicam, reduced proliferation and apoptosis of human colon cancer cells and changed the cell cycle phase distribution. However, they concluded that the ability

of NSAIDs to reduce the rate of proliferation of these colon cancer cells were independent of the PG synthesis pathway. [99]

From our studies, we have some limited data that may suggest that the absence of COX-2 decreased the number of dead cells. Assuming that living cells would adhere to the bottom of the wells but that dead cells would be floating in the media, we collected medium from the cultures and measured the floating cell count. We found that there was twice the number of cells in the medium of the WT cultures compared to the KO, suggesting that there was double the number of dead cells in the WT cultures.

To determine the cause of the increase in cell number in the COX-2 KO cultures, further studies on the role of apoptosis in COX-2 KO cells is necessary. In order to evaluate cellular apoptosis, three techniques may be used: 1) measurement of DNA content by propidium iodide staining and flow cytometric analysis; 2) acridine orange staining; and 3) agarose gel electrophoresis of genomic DNA. [99]

It is also possible that expression of COX-2 simply delays cells in G_1, as suggested by Dubois. [62] Thus, COX-2 KO cells would simply progress more rapidly through mitosis. In order to see if a delay in the cell cycle contributed to the difference in cell count, it would be necessary to synchronize the cell cycles of the cells. Serum-depriving the cells can place them in a quiescent state. Once serum was introduced, the cells should theoretically begin the cell cycle at the same point. If TDR was measured at frequent intervals, as often as every hour, the rates of replication of the WT and KO cells can be measured and any delays in a cell cycle can be established.

Endogenous PGs also had major effects on the differentiation of osteoblastic cells. The absence of COX-2, and consequently measurable PGE_2, inhibited the differentiation

of cells as measured by ALP staining at the end of the first week of culture. We also found that, at the end of the first week of culture, the levels of ALP activity in the WT cultures were 10-17 times greater than in the KO. The difference in differentiation, as measured by ALP staining, appeared to disappear by about day 9. In one experiment, the differences in ALP activity between the WT and KO were no longer significant by day 14 but there was still about a 25% decrease in ALP activity in the KO compared to the WT.

The addition of exogenous PGE_2 (10^{-6} M) to the KO cultures appeared to reverse or speed up the differentiation of the KO cells to the level of the WT. However, the addition of PGE_2 to the WT cultures almost tripled ALP activity levels compared to the untreated WT cultures. Therefore, PGE_2 partially but significantly reversed the delay in differentiation of the KO cells. In contrast to the above results, adding PGE_2 (10^{-7} M) showed no increase in differentiation of the KO cultures at day 6, as measured by ALP staining. This suggests that the effects of PGE_2 on differentiation may be related to its concentration. It is also interesting to note that while PGE_2 (10^{-7} M) corrects the elevated cell count in the KO cultures, it takes a higher concentration of PGE_2 (10^{-6} M) to correct the delay in differentiation of the KO cells.

The effects of PGs in cells are enormously complex thus making it very difficult to study. One reason is that there is uncertainty about which endogenous PGs are produced in addition to PGE_2 by COX-2 WT cells. Therefore, simply adding back PGE_2 may not compensate for the missing PGs. There are also different receptors for different PGs, and hence, different PGs can stimulate different pathways. Finally, COX-2 might have effects independent of PG production as noted above. [99]

Studies done in our lab showed that the bone histology of 5-week-old COX-2 KO mice revealed no skeletal abnormalities. These results suggest that COX-2 is not necessary for normal bone or skeletal development because the circulating levels of maternal PGs may be sufficient to compensate. However, at 3-5 months of age, our studies suggest that there is a reduction of bone mass in mice with deficient COX-2 relative to wild type mice. [100] Thus the absence of COX-2 appears to have an impact on bone turnover bone. Studies in our lab have also shown that PGs produced by COX-2 are potent stimulators of bone resorption and required for maximal stimulation of osteoclastogenesis by other agonists. [100] Hence, in vivo, deficient COX-2 may decrease bone formation more than bone resorption.

Significance

Orthodontics involves the movement of teeth through bone. Biologically, tooth movement is an inflammatory process in which the alveolar bone surrounding the tooth is remodeled in such a way that the bone on the surface under pressure is resorbed and the bone on the surface under tension is deposited. The placement of forces, in an attempt to control the areas of resorption and deposition, is what allows the teeth to move through the bone into a final esthetic position. This remodeling of bone is an intricate and complex process, which is controlled by an array of mediators.

Over the years, orthodontics, as in other medical and dental fields, has evolved for the better. Advancing technology has produced appliances and new materials that have improved results and reduce treatment time. One example of such an advance is the nitinol wire, which has tremendously simplified the process of aligning and leveling. However, there may be a biological threshold beyond which bone remodeling, and therefore tooth movement, cannot occur more quickly. Yamasaki et al. [7, 8] have shown that the rate of orthodontic tooth movement increases with local injections of PGE_2 in monkeys and local injections of PGE_1 in humans. In addition, PG antagonists have been shown to reduce the rate of tooth movement by inhibiting the increase of osteoclasts on the resorptive surface [6] and the differentiation of osteoblasts on the bone-forming surface. [101] Consequently, the future of orthodontics may lie in improving the rate of bone remodeling. The subsequent reduction of treatment time may potentially minimize side effects of orthodontic treatment, such as periodontal disease, enamel demineralization, and root resorption. In addition, a more complete understanding of

how prostaglandins regulate bone resorption and formation may lead to the development of new therapies.

Tables

Table 1. Comparison of body weights and serum creatinine levels from mice with both COX-2 alleles intact (WT), one allele intact (HT) or both alleles disrupted (KO). Adapted from Okada et al. [103]

Genotype (n)	Body weight (gm)	Serum Creatinine (mg/dl)
COX-2 Wild Type (WT)	16.7 ± 1.7	0.93 ± 0.02
COX-2 Heterozygous (HT)	16.6 ± 0.9	0.93 ± 0.01
COX-2 Knockout (KO)	15.1 ± 1.9	0.96 ± 0.04

Data are means ± SEM for 5-wk old C57BI/6 X 129 mice. Each group includes 2 males and 2 females from 2 litters.

Table 2. Summary of Cell Count (cells/ml/well) Data for Wild Type (WT) and COX-2 Knockout (KO) Calvarial Osteoblastic Cultures

		D5		D6		D7		D8	
		WT	KO	WT	KO	WT	KO	WT	KO
EXPT 14168	C	482,667 ± 40,175	885,067 ± 66,740 [a] (1.8 X)	819,400 ± 36,418	1,150,000 ± 55,076 [a] (1.4 X)	-	-	-	-
Donor age: 12 wks	PGE_2 (10^{-7} M)	405,867 ± 39,683	495,133 ± 21,783 [c] (1.2 X)	827,367 ± 50,889 (1.2 X)	717,267 ± 29,459 [c]	-	-	-	-
EXPT 14261	C	-	-	39,900 ± 1,914	178,800 ± 10,024 [a] (4.5 X)	123,733 ± 15,623	256,400 ± 19,713 [a] (2.1 X)	-	-
Donor age: 5 wks	PGE_2 (10^{-6} M)	-	-	50,600 ± 3189.6	127,133 ± 6,664 [c] (2.5 X)	115,167 ± 12,679	160,633 ± 19,189 [d] (1.4 X)	-	-
EXPT 14369	C	-	-	-	-	-	-	55,492 ± 4,987	214,275 ± 49,248 [b] (3.9 X)
Donor age: 51/2-8 wks	PGE_2 (10^{-6} M)	-	-	-	-	-	-	44,825 ± 482	120,258 ± 13,781 (2.7 X)
EXPT 14533	C	248,933 ± 9,624	450,267 ± 13,734 [a] (1.8 X)	386,133 ± 7,585	479,933 ± 21,776 (1.2 X)	-	-	-	-
Donor age: 7 wks	PGE_2 (10^{-6} M)	257,000 ± 22,690	396,067 ± 23,299 (1.5 X)	431,733 ± 38,826	502,933 ± 22,822 (1.2 X)	-	-	-	-

Data are means and SEM for n=3

Data in paranthesis are fold increase in cells/ml/well compared to opposite group

[a] Significant difference from WT, $P < 0.01$

[b] Significant difference from WT, $P < 0.05$

[c] Significant difference from KO, $P < 0.01$

[d] Significant difference from KO, $P < 0.05$

Figures

Figure 1: Adapted from Soma et al. [2] Orthodontics tooth movement. A) Placement of a force produces 0.2-0.3 mm of initial tooth movement due to the compression of the periodontal ligament (PDL). B) Necrosis of the PDL (shaded areas) and undermining bone resorption occurs. Tooth movement is arrested because of the lack of bone resorption in the PDL. C) After the removal of necrotic tissue and completion of undermining resorption, the tooth moves continously by bone resorption. The arrow indicates the direction of the force and tooth movement.

Figure 2: Adapted from Stein et al. [15] Model of the relationship between osteoblastic proliferation and differentiation. There are three sequential stages through which osteoblastic precursor cells progress into a mature osteoblast. As proliferation of the osteoblastic precursor cells decrease, differentiation begins. Markers of osteoblastic differentiation such as Type I collagen, alkaline phosphatase, and osteocalcin begin to be expressed.

Figure 3: Adapted from Burger and Klein-Nulend [25]. Model for mechanotransduction in bone. Stress (large arrows) placed on the mineralized matrix (MM) produce strain that squeeze fluid through the lacuno-canalicular system (small arrows). The network consists of osteocytes (OCY) located in the MM and osteoblasts (OB) on the bone surface by cell processes and gap junctions.

Figure 4: Adapted from Embery et al. [104] Periodontal ligament. The periodontal ligament (PDL) is a connective tissue interface between a tooth and its surrounding bone.

Figure 5: Cell cycle for orthodontically activated fibroblast-like cells in the periodontal ligament of a rat molar [106]. When an orthodontic load widens the periodontal ligament (PDL), quiescent cells located at the end of G_1 (also known as G_0) and G_2, are recruited into the cell cycle to proliferate. G_1 is the pre-synthetic phase. DNA is replicated in the S phase. G_2 is the post-synthetic phase. Cells divide in the M or mitotic phase. Some of these cells differentiate to become osteoblasts while other less differentiated cells continue though the cell cycle.

Figure 6: Prostaglandin production pathway. Cyclooxygenase (COX) converts arachidonic acid (AA) from the membrane bilayer to PGH2 in a cyclooxygenase and peroxidase reaction. PGH_2 is then converted into various prostaglandins by a number of different tissue specific intracellular enzymes. Two forms of COX, COX-1 and COX-2 exist. Although they are 60% homologous and catalyze the same reaction, they are encoded by different genes and are expressed and regulated differentially.

Figure 7: Inhibition of the prostaglandin pathway by non-steroidal anti-inflammatory drugs (NSAIDs) [86]. NSAIDs act by preventing the conversion of arachidonic acid to PGH2 by cyclooxygenase (COX) and thus preventing the production of prostaglandins.

Figure 8: Cell count (cells/ml/well) in COX-2 wild type (WT) and knockout (KO) primary calvarial cell cultures. Data are means ± SEM for 3 wells. [a] Significant difference from WT, $P < 0.01$.

Figure 9: Cell count (cells/ml/well) in COX-2 wild type (WT), heterozygous (HT), and knockout (KO) primary calvarial cell cultures. Data are means ± SEM for 3 wells. [a] Significant difference from WT, $P < 0.05$. [b] Significant difference from HT, $P < 0.01$

Figure 10: PGE_2 (nM) in the medium of COX-2 wild type (WT) and knockout (KO) primary calvarial cell cultures. Fresh medium was added after the media was collected for measurement on day 3. PGE_2 was measured by RIA. Bars are means ± SEM of 3 wells. UD: undetectable

Figure 11: Effects of exogenous PGE_2 (10^{-6} M) on cell count (cells/ml/well) in COX-2 wild type (WT) and knockout (KO) primary calvarial cell cultures. PGE_2 was added at the beginning of culture and at each medium change. Data are means ± SEM for 3 wells. [a] Significant difference from WT, $P < 0.01$. [b] Significant effect of PGE_2 (10^{-6} M), $P < 0.01$.

Figure 12: Effects of exogenous PGE_2 (10^{-7} M) on cell count (cells/ml/well) in COX-2 wild type (WT) and knockout (KO) primary calvarial cell cultures. PGE_2 was added at the beginning of culture and at each medium change. Data are means ± SEM for 3 wells.

[a] Significant difference from WT, $P < 0.05$. [b] Significant effect of PGE_2 (10^{-7} M), $P < 0.01$.

Figure 13: Comparison of DNA content and ^{3}[H]-thymidine incorporation in COX-2 wild type (WT) and knockout (KO) primary calvarial cell cultures on day 7 of culture. Data are means ± SEM of 3 wells. [a] Significant difference from WT, $P < 0.01$.

Figure 14: Effects of exogenous PGE_2 (10^{-6} M) on DNA content and ^{3}[H]-thymidine incorporation in COX-2 wild type (WT) and knockout (KO) primary calvarial cell cultures on day 5 of culture. PGE_2 was added at the beginning of culture and at each medium change. Data are means ± SEM for 3 wells. [a] Significant difference from WT, $P < 0.01$. [b] Significant effect of PGE_2 (10^{-6} M), $P < 0.01$.

Figure 15: Comparison of alkaline phosphatase (ALP) staining of COX-2 wild type (WT) and knockout (KO) primary calvarial cell cultures. Cells were grown for 6 and 9 days before staining.

Figure 16: Effects of exogenous PGE_2 (10^{-6} M) on alkaline phosphates (ALP) activity in COX-2 wild type (WT) and knockout (KO) primary calvarial cell cultures. PGE_2 was added at the beginning of culture and at each medium change. ALP activity was measured for days 7 and 14 in two different experiments. Data are means ± SEM for 3 wells. [a] Significant difference from WT (treated in corresponding manner), $P < 0.05$. [b] Significant effect of PGE_2 (10^{-6} M), $P < 0.01$.

Figure 17: Effects of exogenous PGE_2 (10^{-6} M) on alkaline phosphatase (ALP) staining in COX-2 wild type (WT) and knockout (KO) primary calvarial cell cultures on day 7 of culture. PGE_2 was added at the beginning of culture and at each medium change.

Figure 18: Effects of exogenous PGE_2 (10^{-6} M) on alkaline phosphatase (ALP) activity in COX-2 wild type (WT) and knockout (KO) primary calvarial cell cultures. PGE_2 was added at the beginning of culture and at each medium change. ALP activity was measured on days 7 and 14 of culture. Data are means ± SEM for 6 wells. [a] Significant difference from WT (treated in corresponding manner), $P < 0.05$. [b] Significant effect of PGE_2 (10^{-6} M), $P < 0.01$.

Figure 19: Effects of exogenous PGE_2 (10^{-6} M) on alkaline phosphatase (ALP) staining in COX-2 wild type (WT) and knockout (KO) primary calvarial cell cultures. PGE_2 was added at the beginning of culture and at each medium change.

Figure 20: Effects of exogenous PGE_2 (10^{-7} M) on alkaline phosphatase (ALP) staining in COX-2 wild type (WT) and knockout (KO) primary calvarial cell cultures. PGE_2 was added at the beginning of culture and at each medium change.

Figure 1

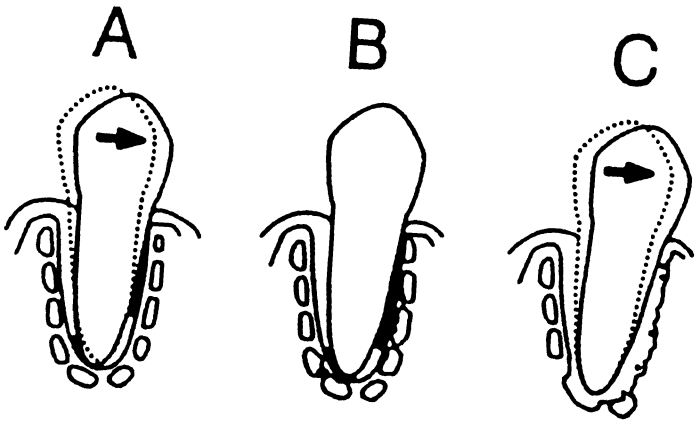


Figure 2

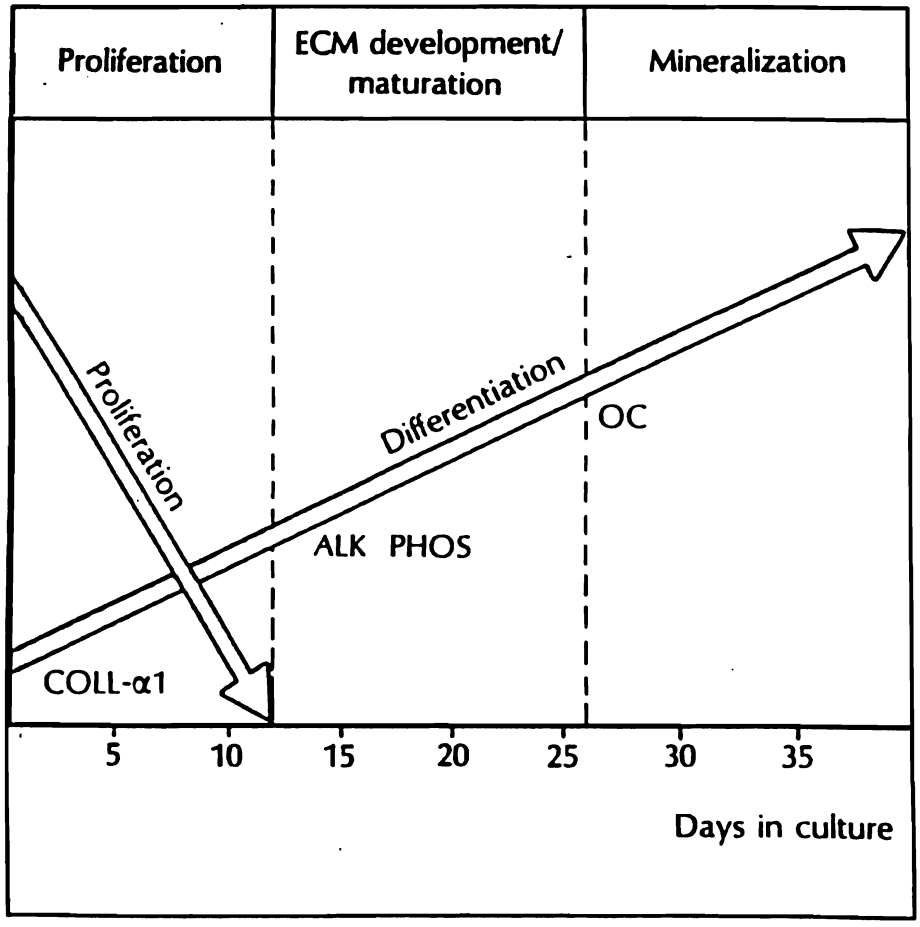

Proliferation
ECM development/ maturation
Mineralization
Proliferation
Differentiation
OC
ALK PHOS
COLL-α1
5
10
15
20
25
30
35
Days in culture

Figure 3

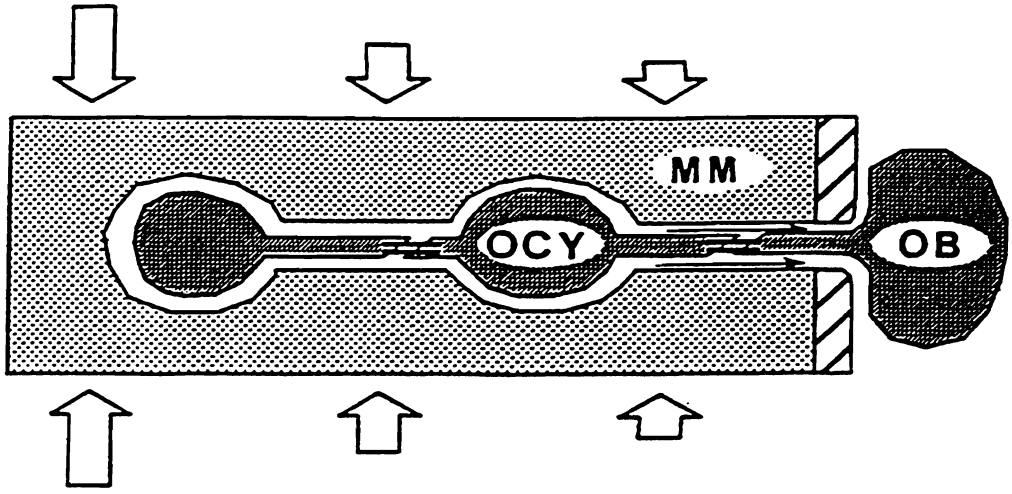


Figure 4

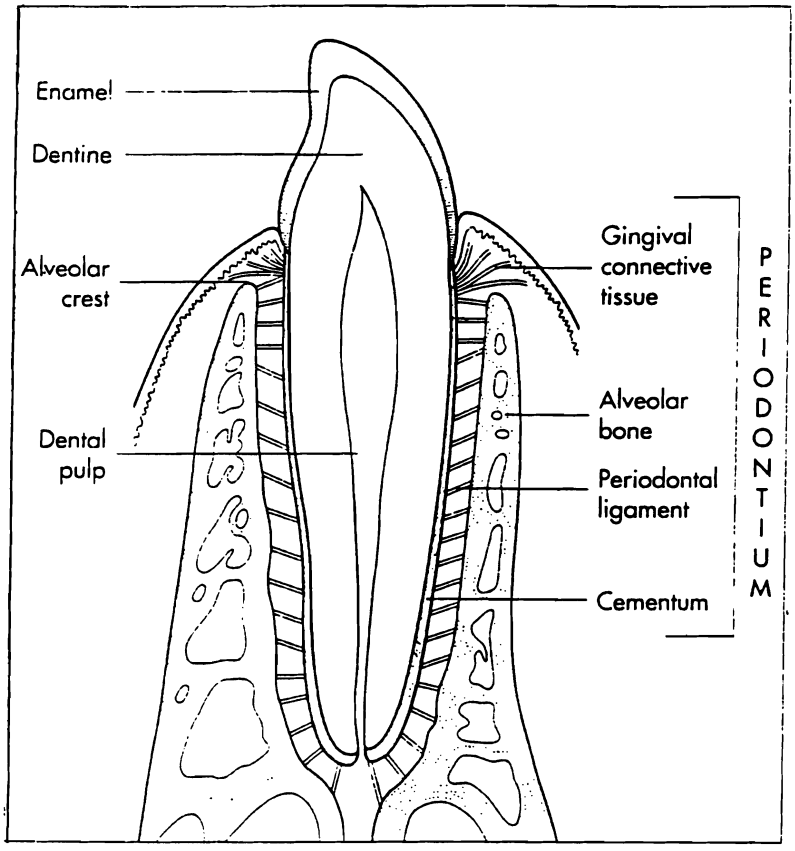

Enamel
Dentine
Alveolar crest
Dental pulp
Gingival connective tissue
Alveolar bone
Periodontal ligament
Cementum
PERIODONTIUM

Figure 5

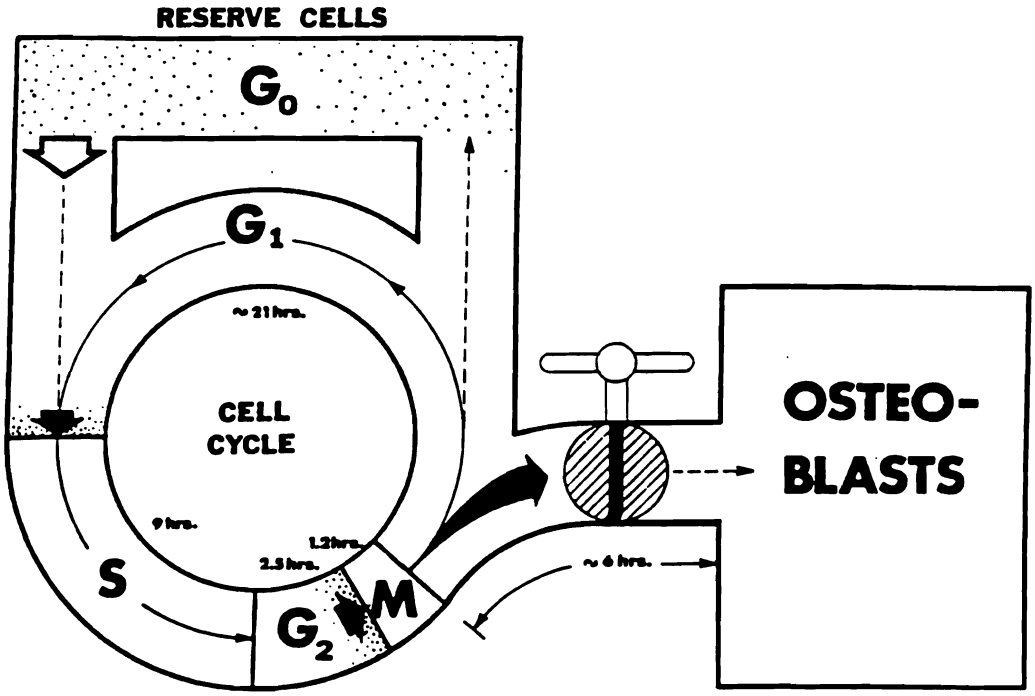
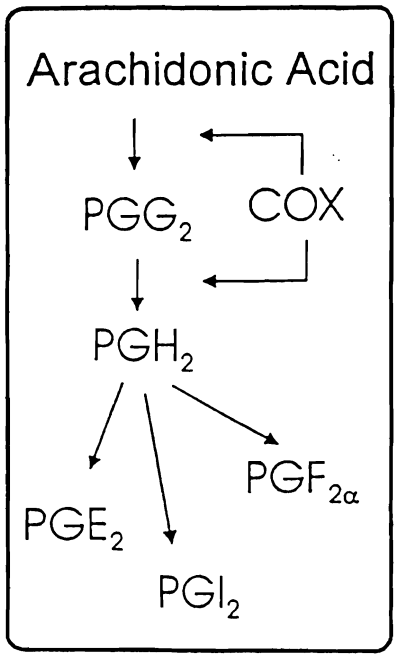

RESERVE CELLS
G_0
G_1
~ 21 hrs.
CELL
CYCLE
9 hrs.
1.2 hrs.
2.5 hrs.
S
G_2
M
~ 6 hrs.
OSTEO-
BLASTS

Figure 6



	COX-2	*COX-1*
Gene (kb)	8	22
Exons	10	11
mRNA (kb)	4.5	2.8

60% homology at nucleic and amino acid levels

70 kDa glycoproteins located in ER and nuclear membranes

Figure 7

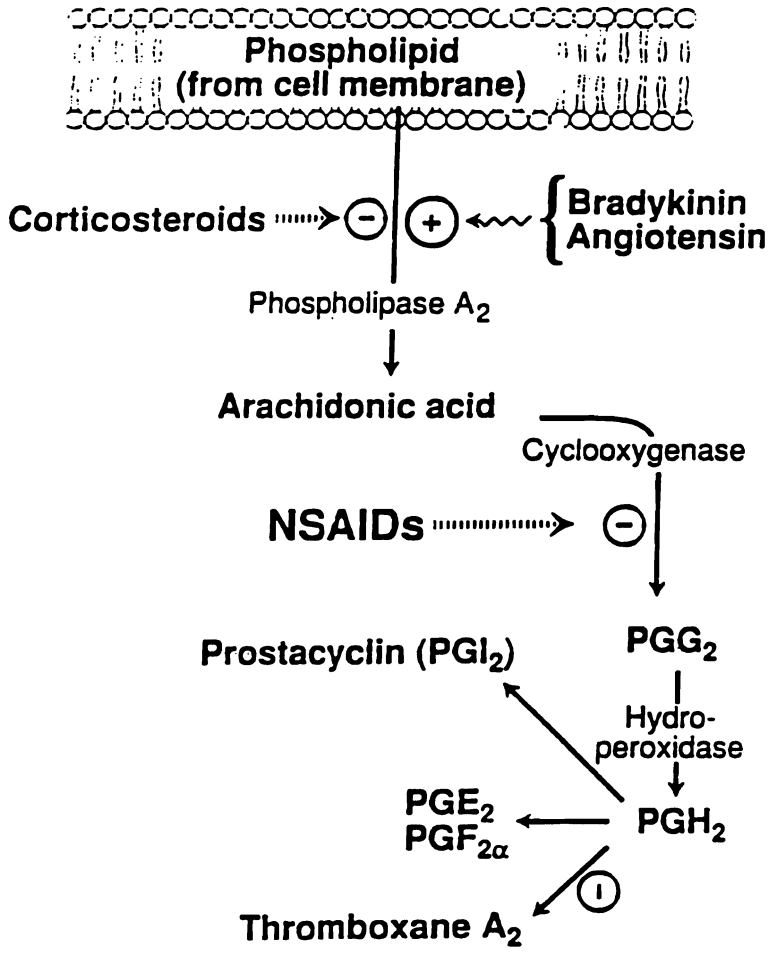

Phospholipid
(from cell membrane)
Corticosteroids
Bradykinin
Angiotensin
Phospholipase A2
Arachidonic acid
Cyclooxygenase
NSAIDs
Prostacyclin (PGI2)
PGG2
Hydro-
peroxidase
PGE2
PGF2α
PGH2
Thromboxane A2

Figure 8

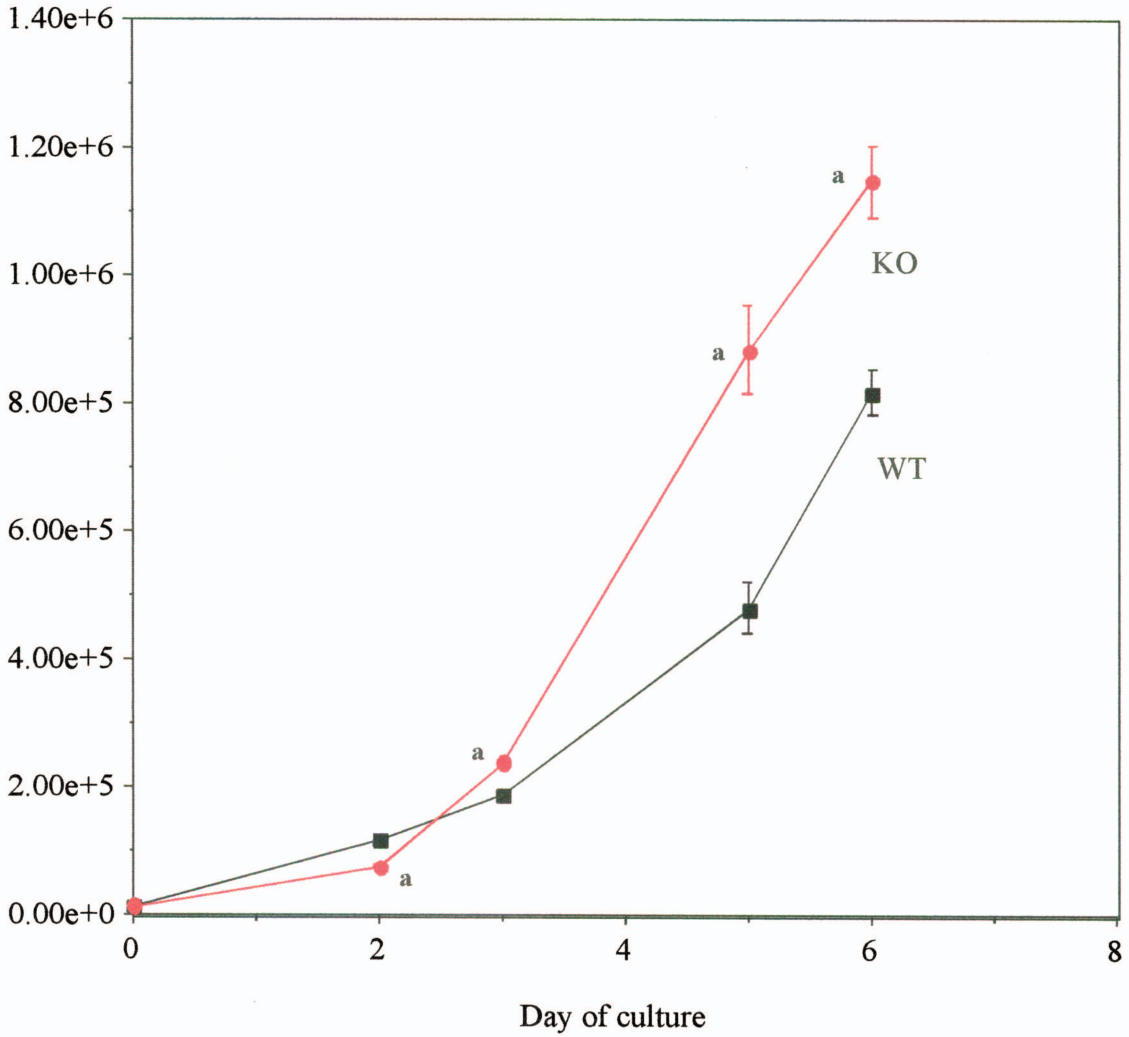

1.40e+6
1.20e+6
1.00e+6
8.00e+5
6.00e+5
4.00e+5
2.00e+5
0.00e+0
Cell count
(cells / ml / well)
0
2
4
6
8
Day of culture
KO
WT
a
a
a

Figure 9

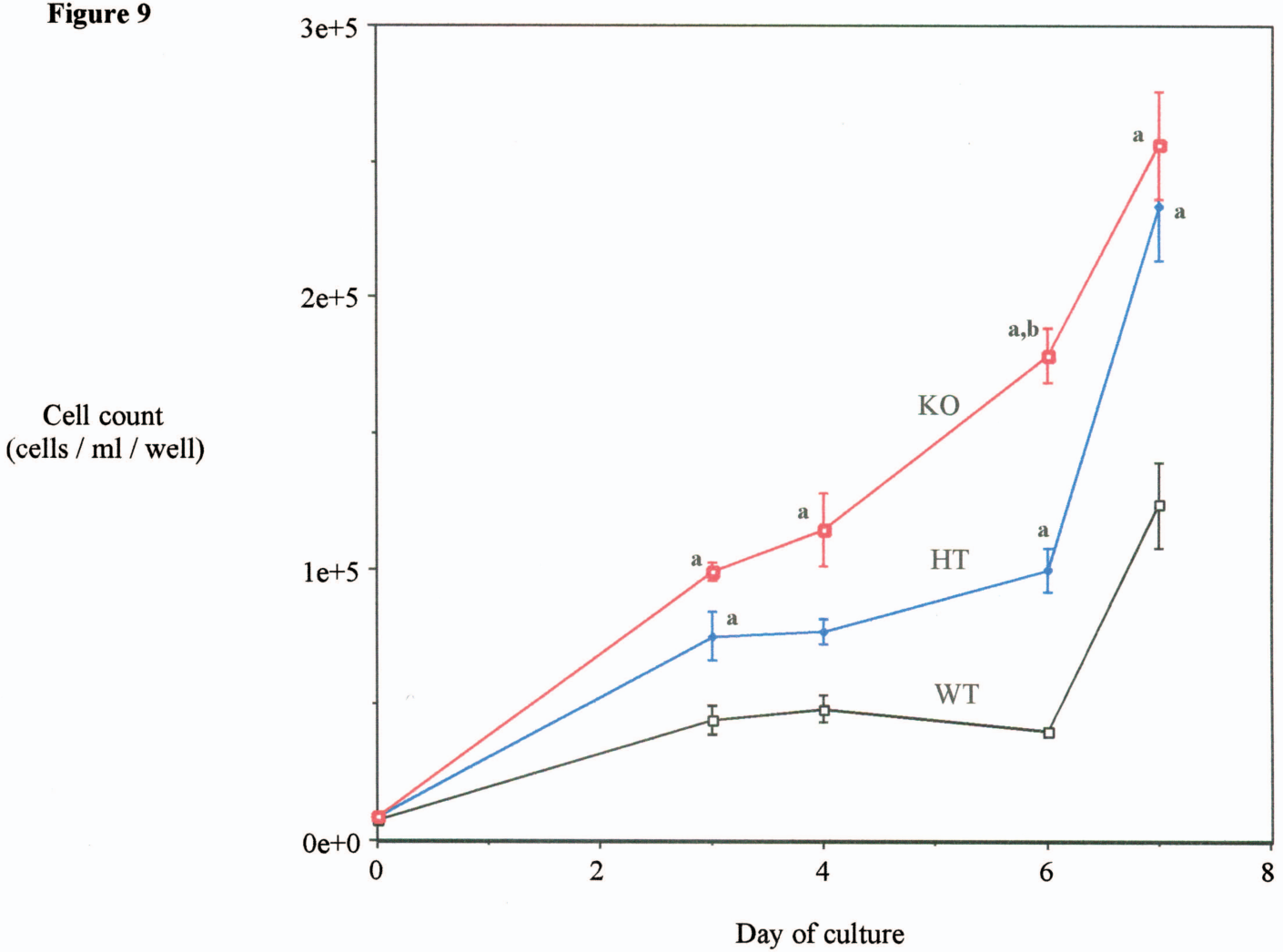

3e+5
2e+5
1e+5
0e+0
0
2
4
6
8
Cell count
(cells / ml / well)
Day of culture
KO
HT
WT
a
a
a,b
a
a
a

Figure 10

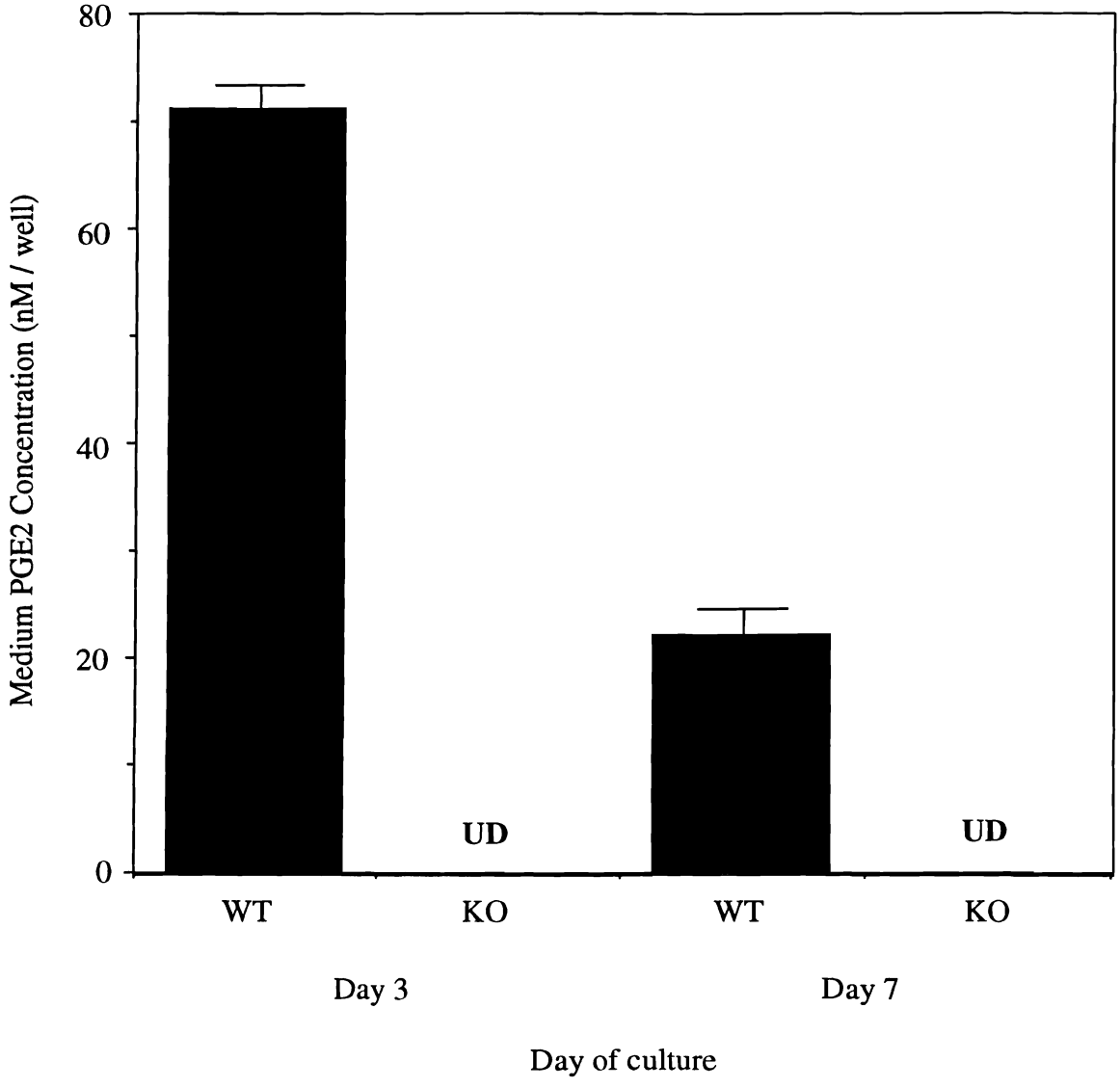

80
60
40
20
0
Medium PGE2 Concentration (nM / well)
UD
UD
WT
KO
WT
KO
Day 3
Day 7
Day of culture

Figure 11

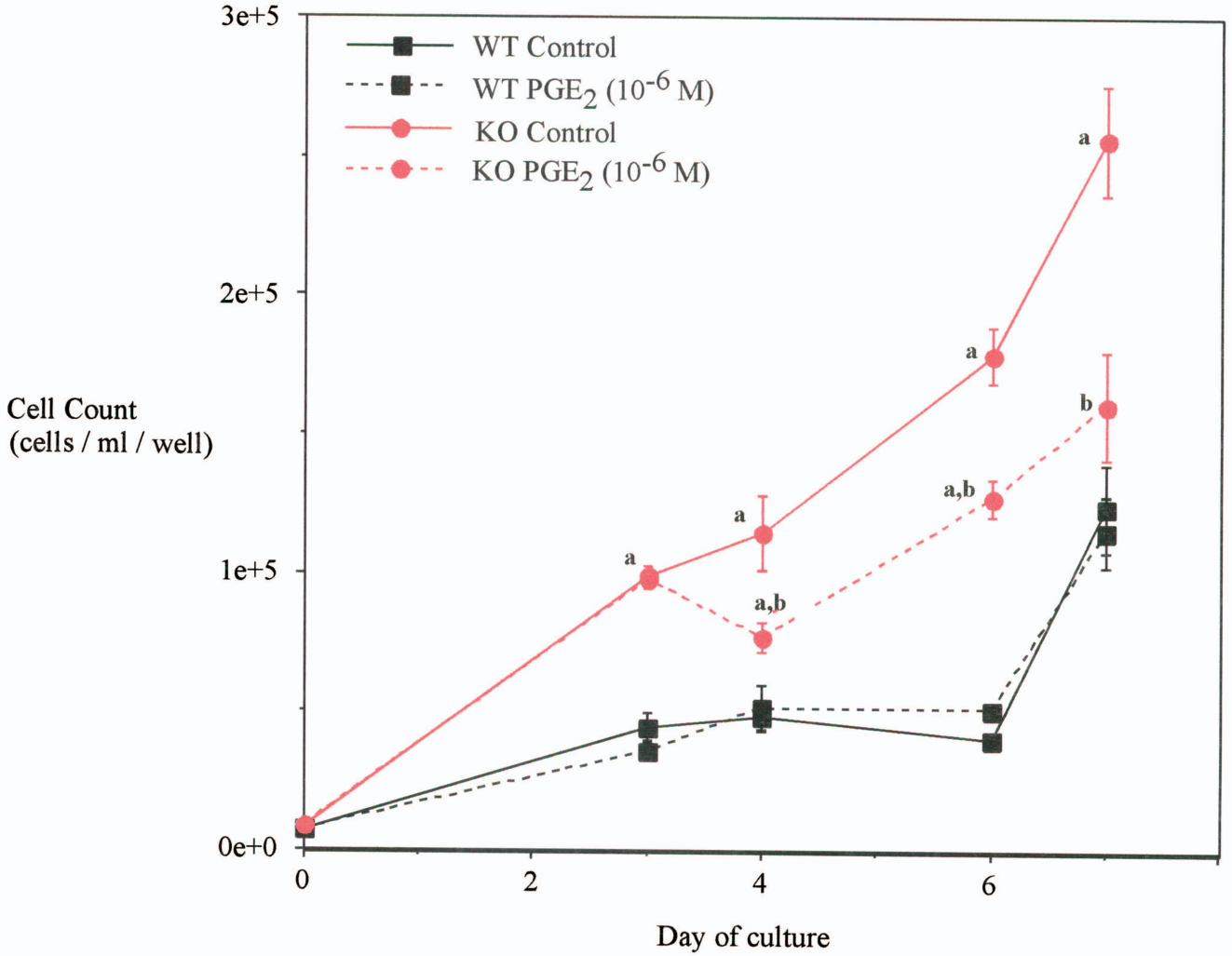

3e+5
2e+5
1e+5
0e+0
Cell Count
(cells / ml / well)
WT Control
WT PGE2 (10-6 M)
KO Control
KO PGE2 (10-6 M)
a
a
b
a,b
a
a,b
0
2
4
6
Day of culture

Figure 12

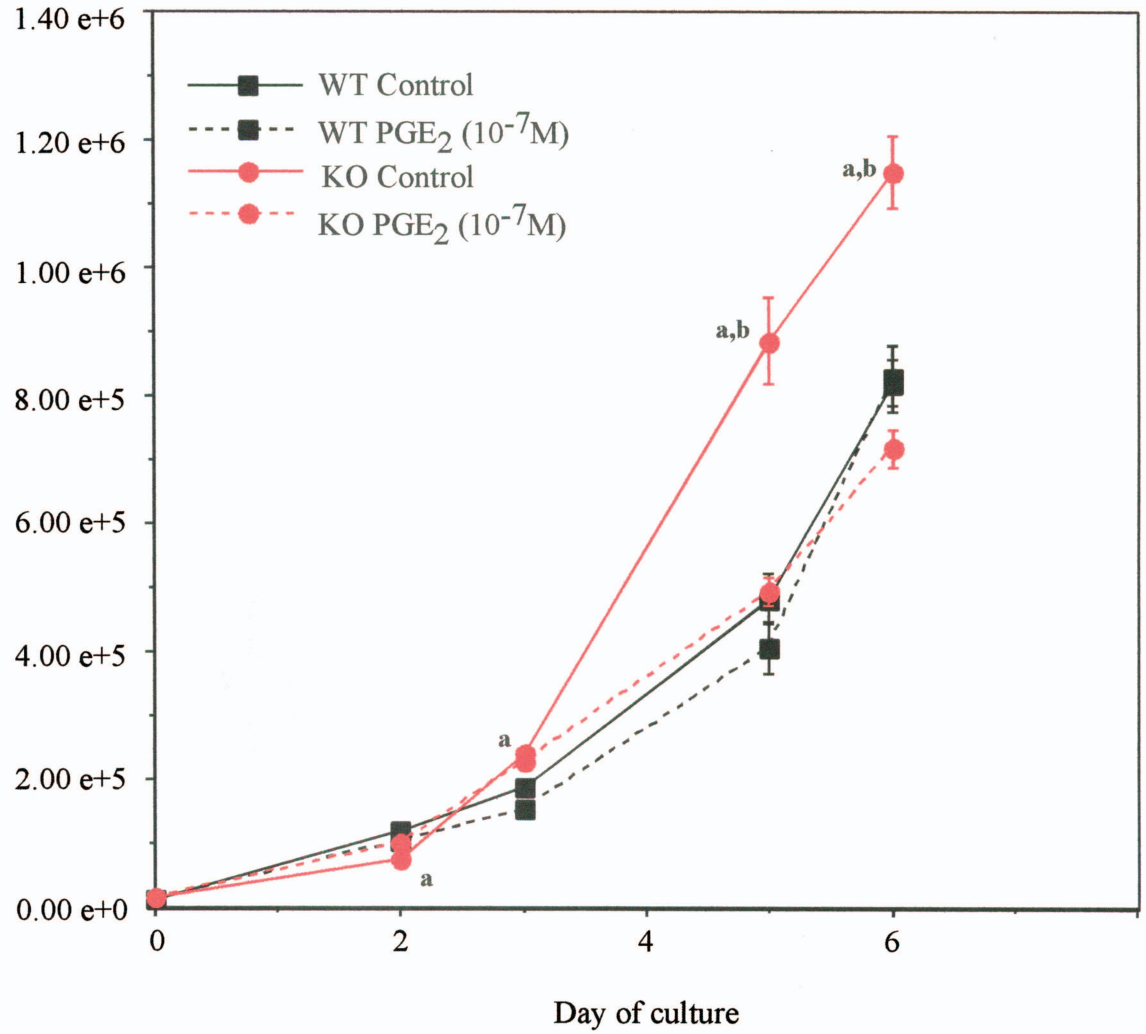
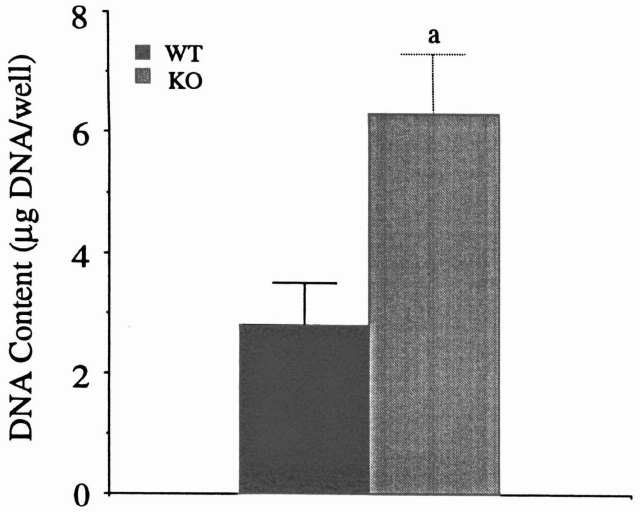
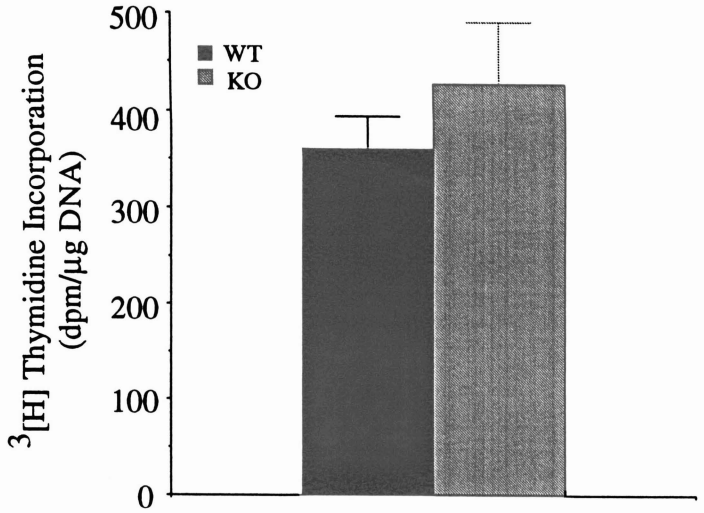

1.40 e+6
1.20 e+6
1.00 e+6
8.00 e+5
6.00 e+5
4.00 e+5
2.00 e+5
0.00 e+0
Cell count
(cells / ml / well)
WT Control
WT PGE2 (10-7M)
KO Control
KO PGE2 (10-7M)
a
a
a,b
a,b
0
2
4
6
Day of culture

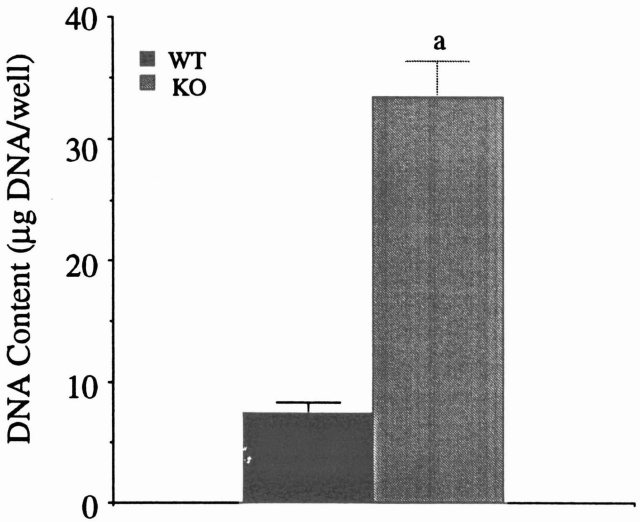
Figure 13

Exp 1





Exp 2



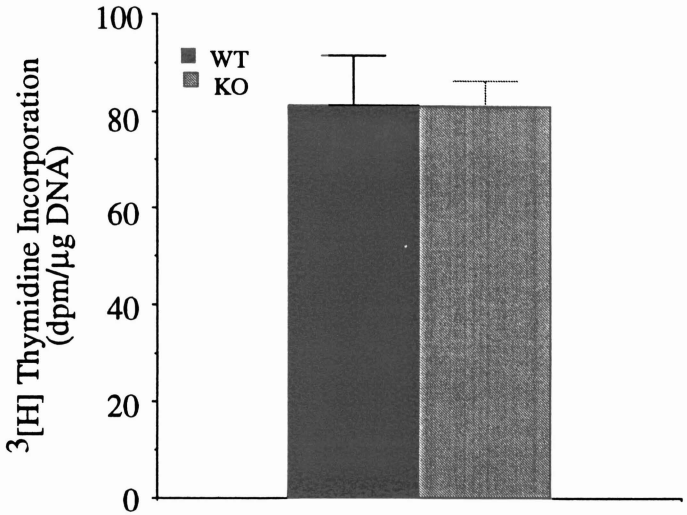


Figure 14

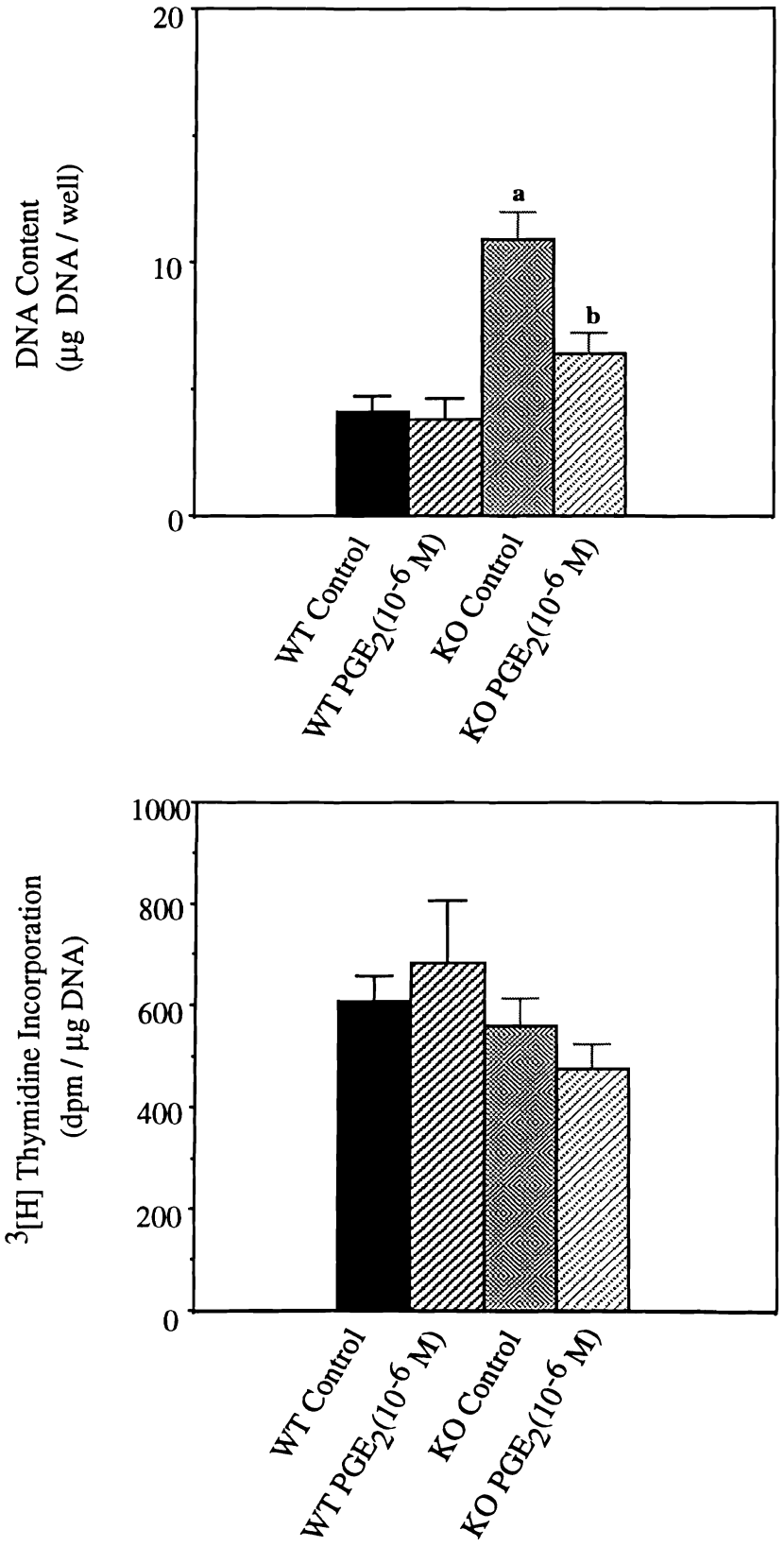

DNA Content
(µg DNA / well)
20
10
0
a
b
WT Control
WT PGE2(10-6 M)
KO Control
KO PGE2(10-6 M)
3[H] Thymidine Incorporation
(dpm / µg DNA)
1000
800
600
400
200
0
WT Control
WT PGE2(10-6 M)
KO Control
KO PGE2(10-6 M)

Figure 15

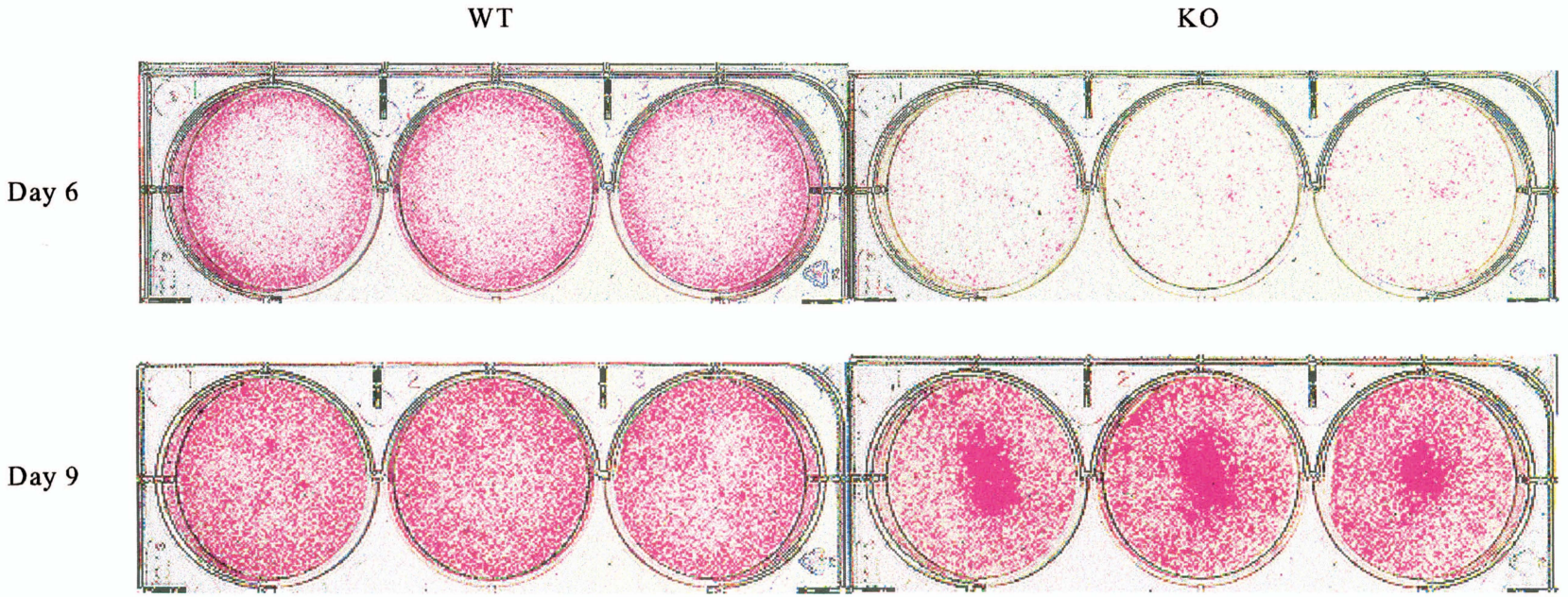
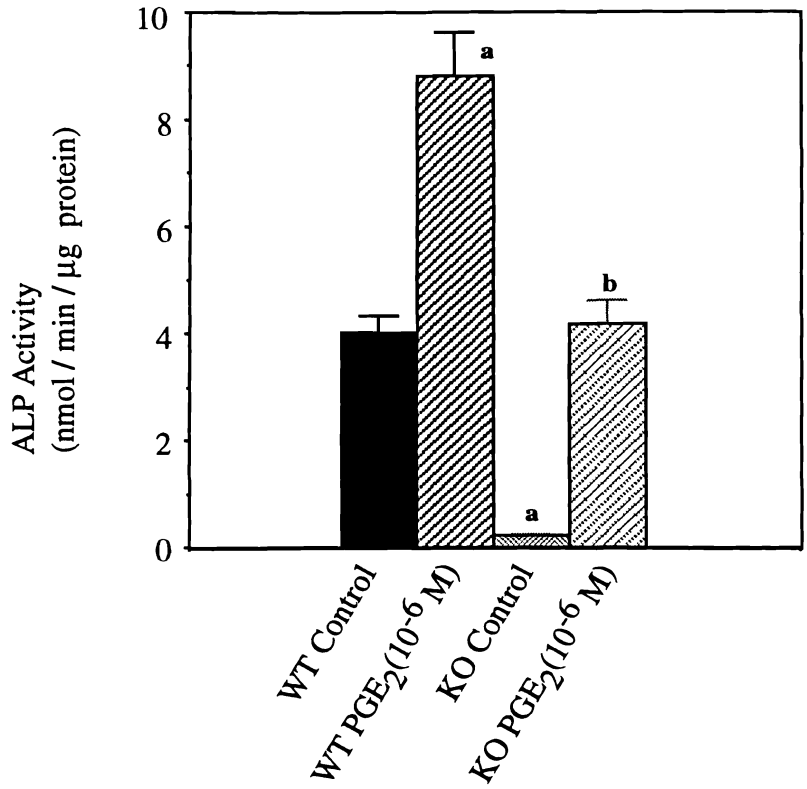


Figure 16

Exp 1
Day 7



Exp 2
Day 14

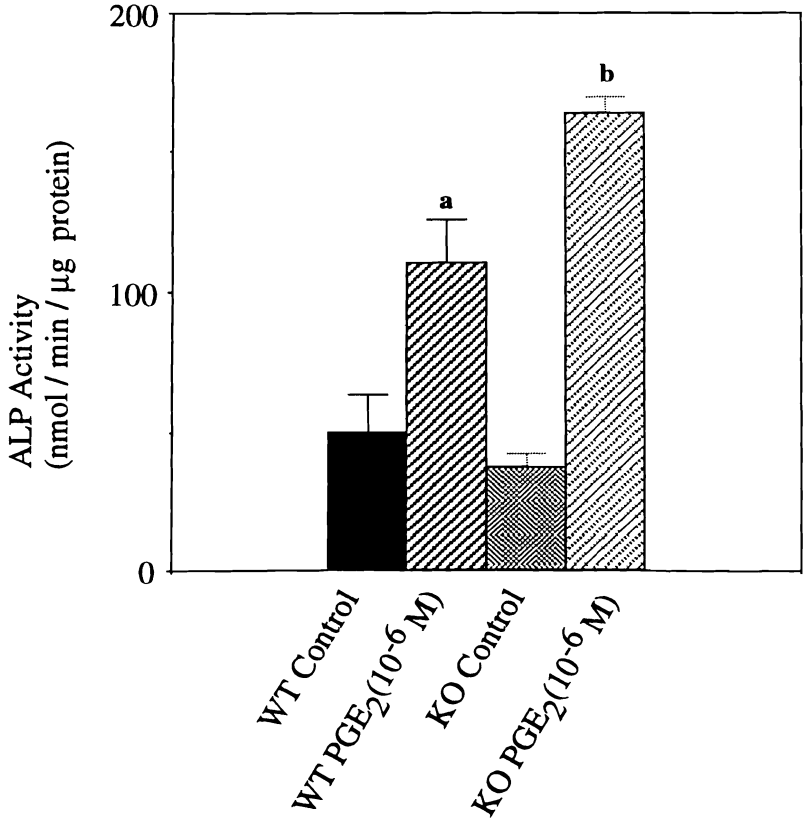


Figure 17

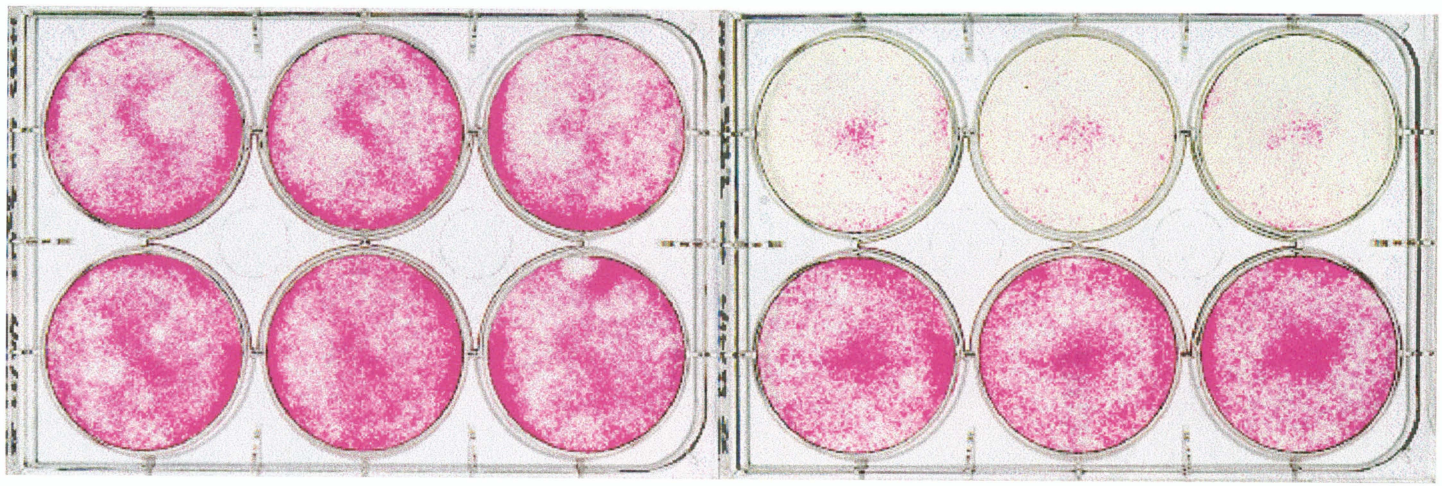
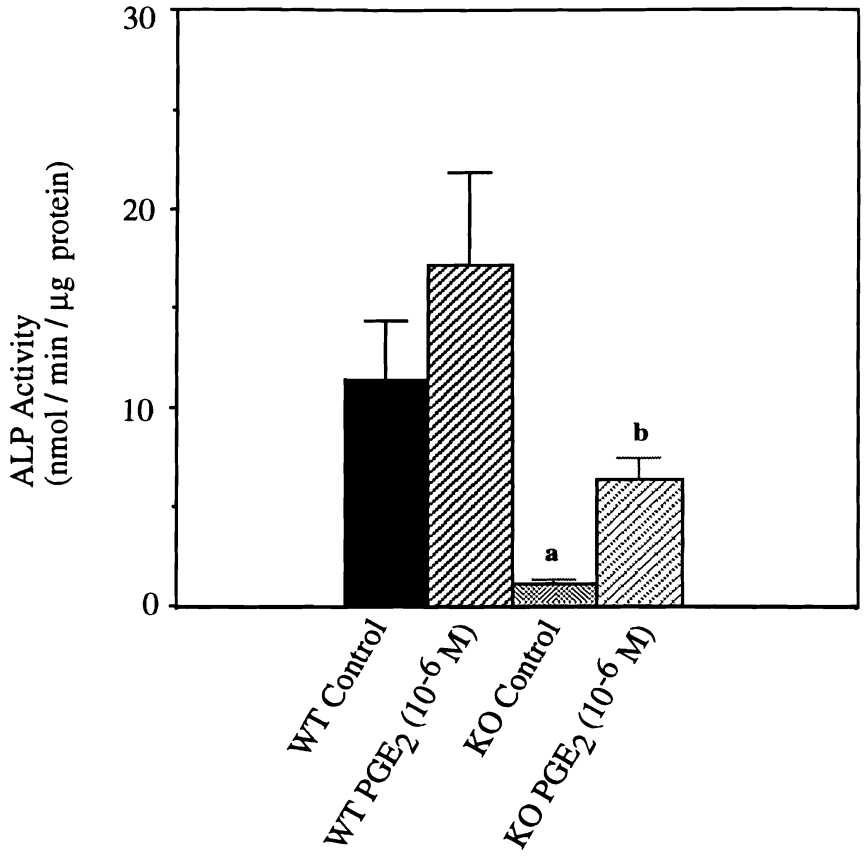


Figure 18

Day 7



Day 14

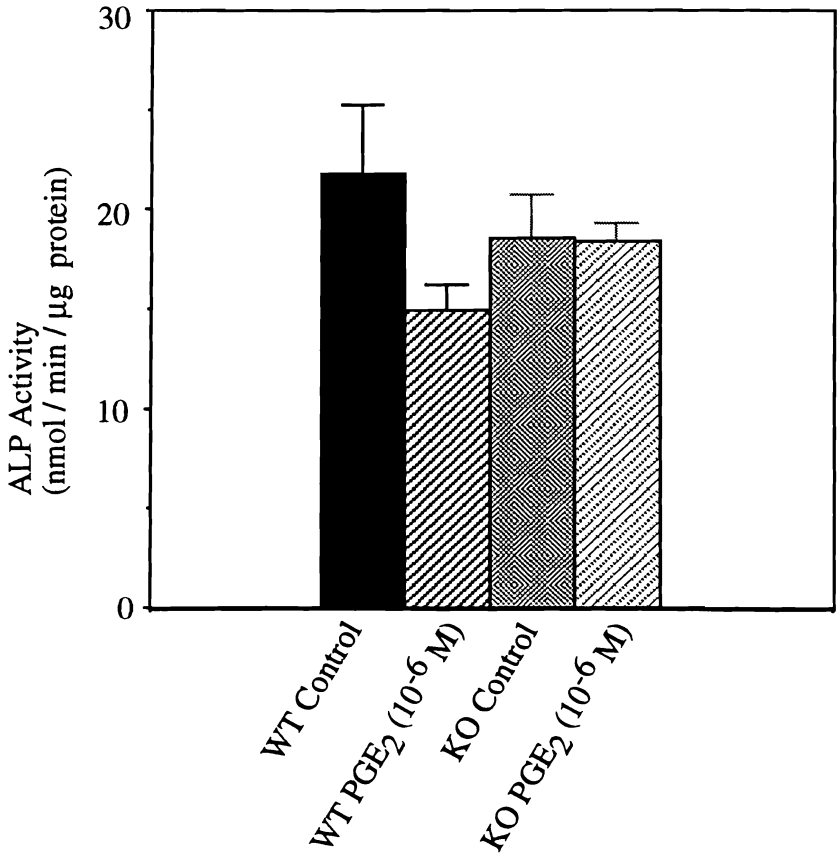


Figure 19

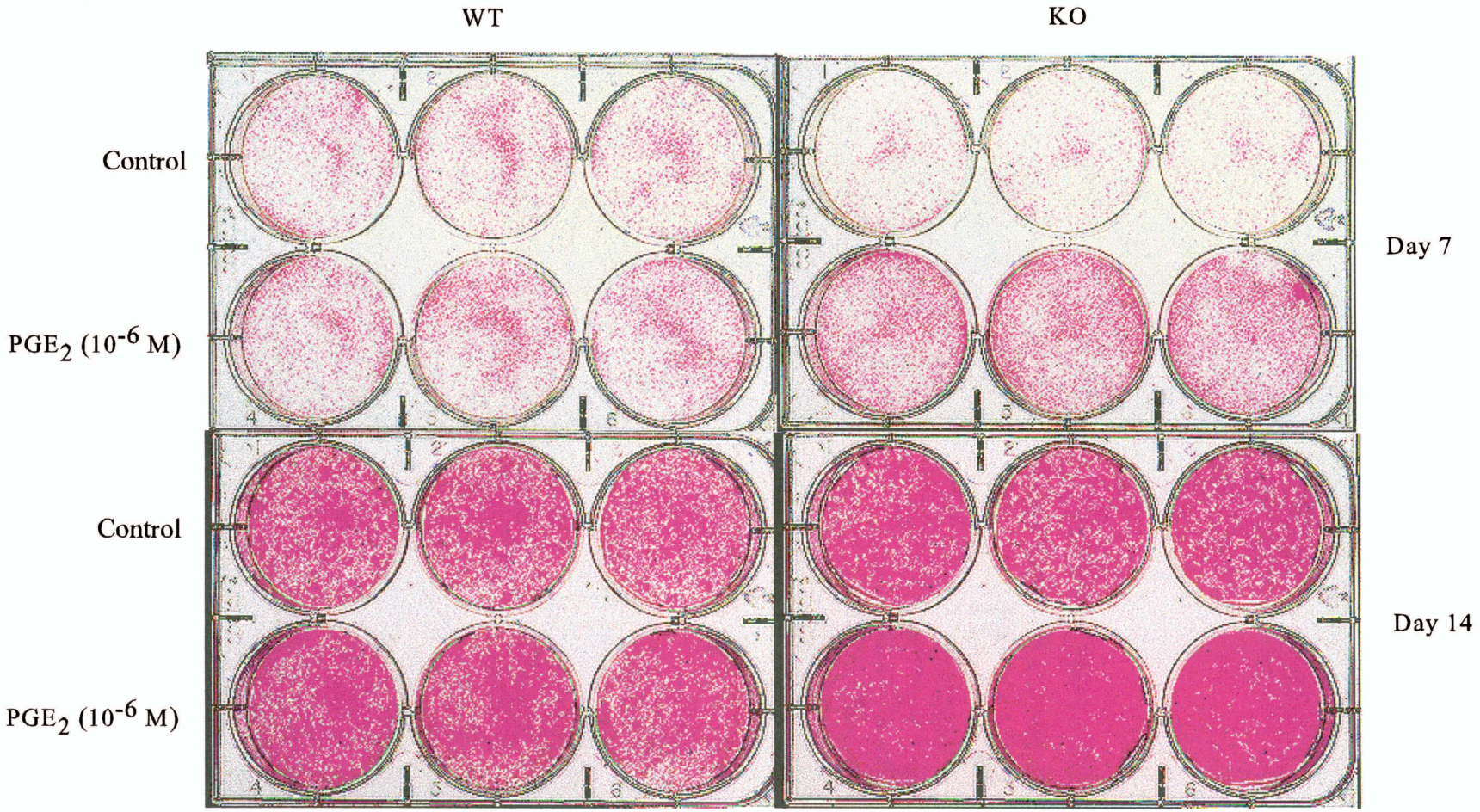
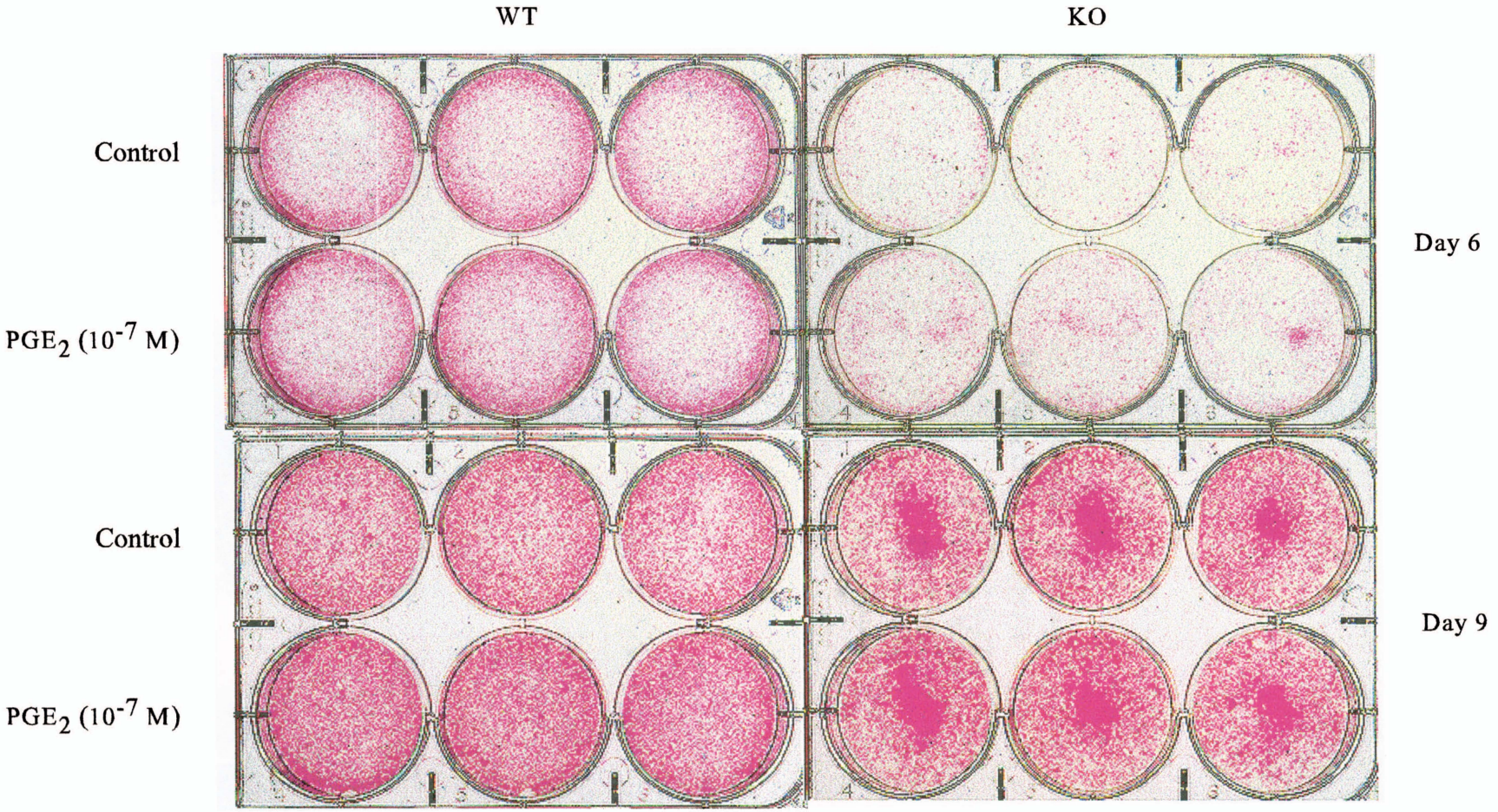


Figure 20



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